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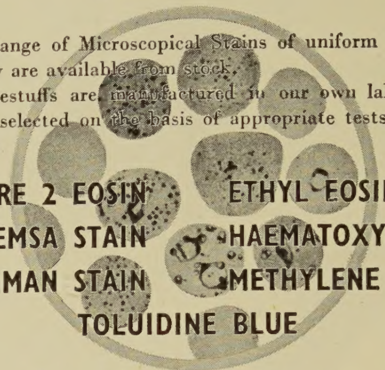

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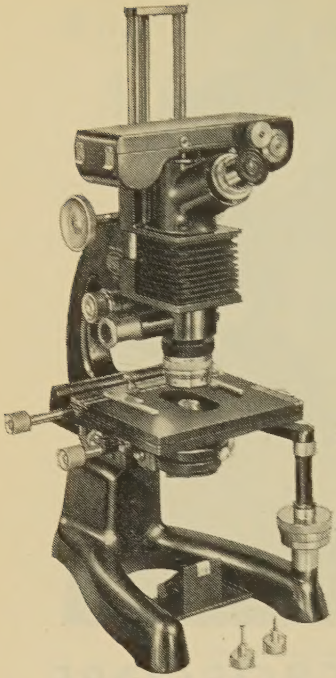

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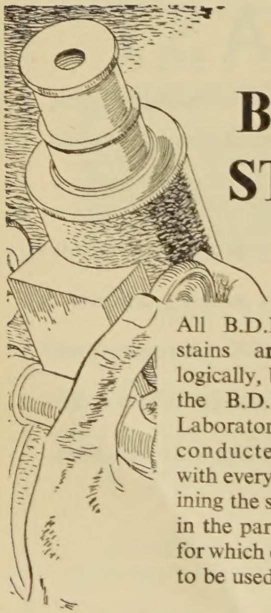

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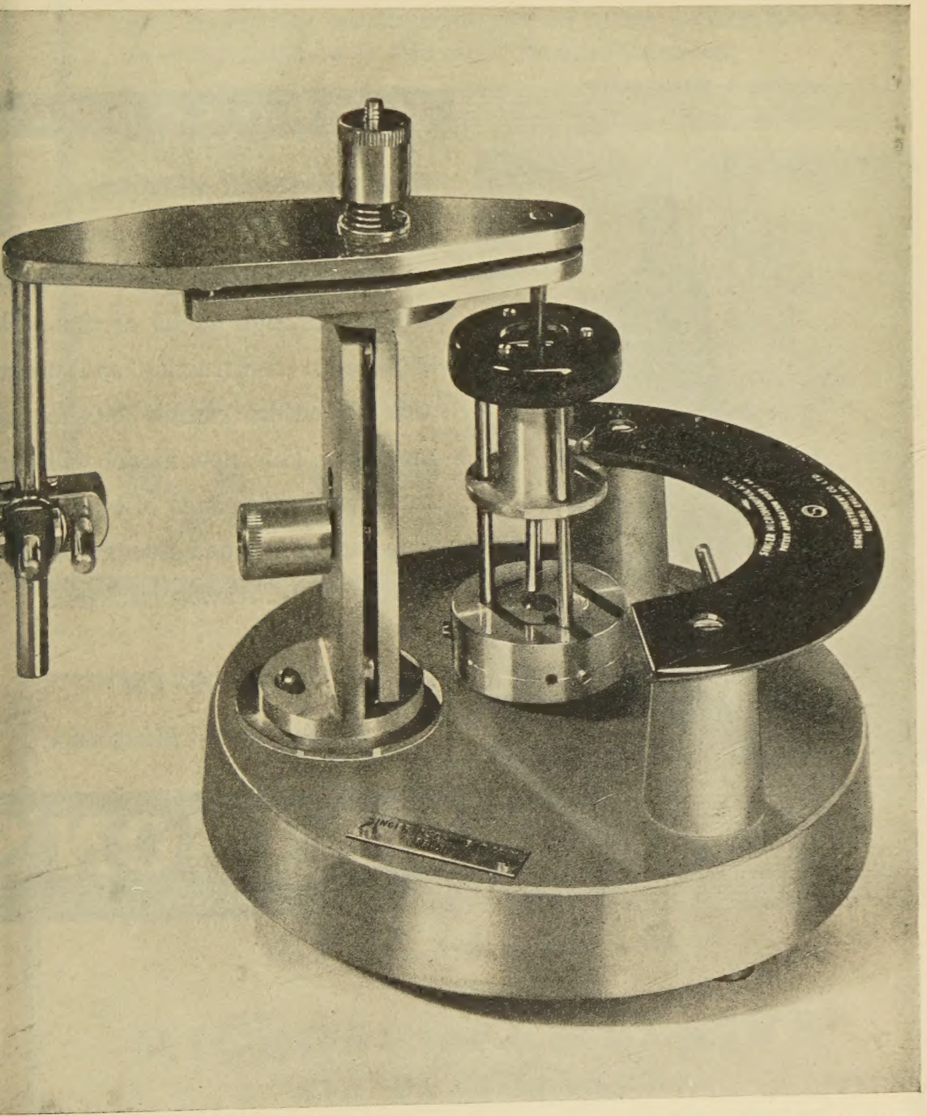

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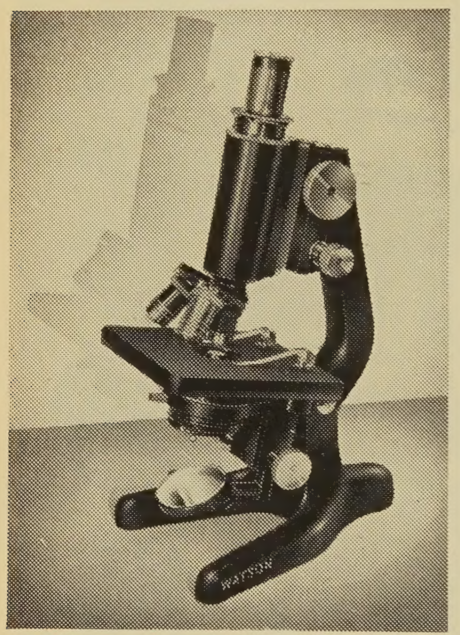

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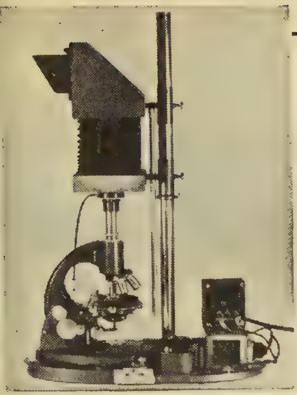

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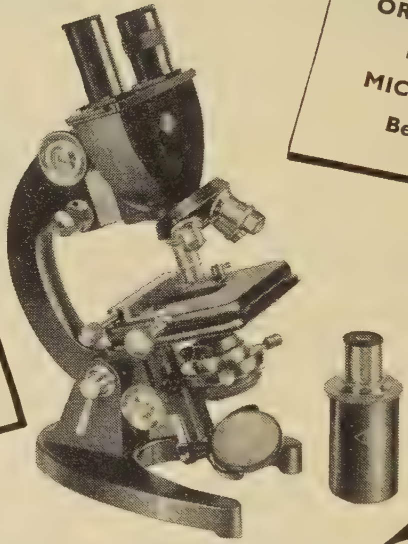

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MICROSCOPICAL SCIENCE

THE *Quarterly Journal of Microscopical Science* has now run for 100 years. How far it is still performing its task to the satisfaction of the public is a matter for editorial self-examination rather than for editorial comment. But a centenary is also an occasion when we can usefully inquire what in fact is the task which a journal such as this should perform. The province of a well-established journal changes with the times, and any historical study of it forcibly reminds us how much our contemporary view of natural knowledge and its divisions is a matter of fashion—a fashion that depends upon attitudes of mind we have inherited from the past. Often it is only when we hear a naturalist refer to 'corrosive sublimate', or a physiologist to 'animals lower in the scale' that we realize how our outlook embodies forgotten, discarded, and even inconsistent ideas; and that what we think today is not just the self-evident modern thing we suppose it to be. A backward look over 100 years can help us to understand why we think as we do today.

Anything that urges us to devote time to consideration of this question is valuable. The great modern endowment of research and the tendency to link professional advancement with quantity of publication has faced the scientific reader with more printed matter than his considered judgement can absorb. And in our own research, technical advance has so increased our capacity to 'do' in the laboratory that we have little time and inclination to sort out the trivial in our work from what is valuable, and still less to follow our problem with new and unfamiliar methods into sciences where we may fear to display our ignorance: it is so easy to continue to turn out papers on the results of our well tried and reliable techniques. The task of considering where our work is leading us is thus much more difficult than ever before.

When we look over the scope of the *Quarterly Journal* since its foundation we see at once how varied have been the aims of scientific work and how greatly scientific fashion has influenced them. The publications in the earliest volumes were essentially descriptive. They reflected the intense interest in the rich variety of new objects and phenomena which the increasing perfection of the light microscope had rendered accessible. With the advent of Edwin Ray Lankester's editorship in the seventies the Journal became transformed into the chief British organ concerned with the rising sciences of comparative anatomy and embryology of the invertebrata, and to a less extent of the vertebrata. The next 50 years were the great period of evolutionary morphology, and much of the classical work which must always remain in our text-books appeared in this Journal. During the editorship of Goodrich between the two world wars, this tradition was maintained—perhaps nowhere more splendidly than in his own work. But the great work of the evolutionary

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morphologists was now done, and the new methods of investigating the living animal given to biologists by the physicists and chemists, diverted research into many new fields.

Since 1945 the outlook in research has again changed. Biochemistry and physical methods like that of electron microscopy have made possible a new morphology, one which can describe finer grades of structure than any previously investigated. But this morphology is by no means only a study of the excessively minute. On the contrary, it is concerned with every grade of organization including that investigated by earlier microscopists. It is a particular virtue of our modern microscopes that they enable us to see the objects in the living cell that previously escaped examination not because they were very small, but because they were very transparent. The worker of earlier days necessarily spent much of his time in studying preparations distorted by fixation and curiously stained. He described his results in terms of a strange para-chemistry, often involving fanciful words devoid of meaning for the chemist. The cytologist or anatomist of today can use the phase-contrast and interference microscopes, and the very sensitive modern polarizing instrument, to obtain new information about the living organism and its parts; and he can then set out to determine the constitution of the objects he sees, by applying the rigorous methods of histochemistry and enzymology.

This new morphology differs from that of the preceding, evolutionary, phase. It is concerned with the machinery of living matter rather than with its phylogenetic origins and systematic relationships. Hand in hand with this, the application of physical and physiological principles to the interpretation of anatomical structure has led to a revival of pre-Darwinian functional morphology in a modern form. The present phase of biology in fact shows a curious resemblance to that at the time of the foundation of the Journal; even the enthusiastic descriptions of objects by the still earlier microscopists have a parallel in the published results of the biophysicists of today. As so often happens, this return to older ideas in a modern setting has been most stimulating. It is not just the re-gleaning of exhausted ground:

> . . . how are our brains beguiled,
> Which, labouring for invention, bear amiss
> The second burthen of a former child!

But such trends do not describe the whole progress of any branch of science. Throughout the history of the Journal new kinds of investigation have continually made their appearance. There were papers on the biochemistry of pigments in the nineties, for instance, and on fertilization, ciliary activity, and tissue culture in the twenties. This tendency to variety can indeed be seen today in this present number. Such new ventures have been of peculiar importance, for they have been forerunners which have led to new kinds of biology.

The publication of these new and unexpected ventures in research raises an important question: what should determine the scope of a scientific journal? We see a continual growth of specialization and specialized journals, with the consequent division of the field of biological publication into an increasing number of restricted compartments. Such specialization has an undoubted convenience. Our mental processes find it easier to deal with ideas if they have first been exactly classified and defined. But a much more powerful factor urging us towards specialization is its great administrative convenience in a busy world.

It would be idle to contest today the many advantages that have come to us from the specialization of important fields of research. But it would also be idle to contest that this specialization carries disadvantages. The ever increasing multiplication of scientific publications is one. A more fundamental disadvantage concerns the nature of scientific research itself. Work in specialized and well-recognized fields of science leads to results largely along predictable lines: we are working out in detail the consequences of some earlier advance. But the most important scientific advances of all have a way of arising in quite another manner, through the intuitive perception of relationships which our specialized categories have placed asunder.

The new ventures in research with which every editor meets from time to time are not always the forerunners of scientific progress. Most ugly ducklings have a great way of developing into ugly ducks. But amongst these new ventures are the occasional few that in due time will transform scientific advance. However administratively convenient it may be to divide the field of scientific publication into defined compartments, it is the duty of every editor, and of those who finance and support him, vigilantly to encourage publication of the new, the unexpected, and the difficult to classify. He must sometimes transgress the supposed limits of his field of publication, and above all he must sometimes suffer gladly work which contemporary opinion may despise.

This admonition is easier to give than to observe. But it does suggest a consequence of some importance about the fields of scientific journals. If specialization we must have, it is better that it should be based upon natural classes of phenomena rather than upon systems of contemporary ideas. Microscopical Science, the field of this Journal, includes just such an acceptable class. The varied biological fashions which it has faithfully reflected in its century, show how independent it is of a purely contemporary outlook. On the other hand, it corresponds to an important division of natural phenomena. For it is characteristic of living things that they are built up from a hierarchy of grades of structural complexity ranging from that of the gross organ-systems of the older morphology down to that of the molecular structure of the substances which compose their bodies. The principles that govern these extreme grades of organization are very different. Those that govern the organ-systems are those brought to light by the evolutionary morphologists and by the pre-Darwinian functional morphologists before them. Homology and adaptation, natural selection, and the mechanics of matter in the mass are among these principles. At the other extreme, molecular structure is governed by the well defined but very different laws of chemistry and molecular physics. But between these grades there are others: those of cellular systems, of the cell itself and of those remarkable cellular constituents whose importance and whose variety is becoming increasingly evident; nuclear structures, mitochondria, and various other cytoplasmic bodies. How far the organization of these intermediate grades is determined by principles similar to those which hold in either the highest or the lowest grades of organization, and how far it may depend on principles of a different sort, we do not know: but it is in just this field that new methods of investigation are now rapidly advancing knowledge. It is the study of these same intermediate grades of organization which comprises Microscopical Science, and however much the fashion of contemporary research may change what we do in the laboratory, all that may concern them falls within the scope of this Journal.

THE EDITORS

New Features of Protoplasmic Structure Observed in Recent Electron Microscope Studies

By J. R. G. BRADFIELD

(*From the Cavendish Laboratory, Cambridge*)

With six plates (figs. 1–6)

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SUMMARY

1. This article discusses current electron microscope specimen techniques (particularly thin sectioning) and various special aspects of protoplasmic structure. It is not a general review and deals only with subjects studied in this laboratory. Dimensions specified are approximate only.

2. As regards sectioning (which is inevitable for the study of fine cell structure in most complex tissues, by whatever microscope) electron microscope practice has two considerable advantages over light microscope practice: (*a*) osmium tetroxide solutions alone (without addition of dichromate, &c.) are excellent for cytoplasmic fixation; (*b*) after cutting, sections receive no further treatment (such as de-waxing and re- and de-hydration). Moreover, criticisms regarding vacuum drying of electron microscope specimens are irrelevant to electron microscope studies of sections. For high resolution, sections must be cut at 0·05 μ or less and examined without removal of embedding medium. Such sections are obtained by embedding in medium-hard plastics and cutting on a glass knife. Specially sharpened blades can be used instead of glass, but it is doubtful whether waxes can be substituted for plastics.

3. Many, possibly all, animal and plant flagella contain two similar central sub-fibrils surrounded by a ring of nine fibrils different in size and chemical composition from the central pair. As far as is known at present, mammalian sperm are unique (*a*) in containing a second concentric ring of nine sub-fibrils and (*b*) in possessing a double-helix sheath round the axial sub-fibrils of the tail. Bacterial flagella consist of single fibrils each equivalent to one of the component sub-fibrils of a multi-fibrillar flagellum. They often occur in bunches, but so far no intermediates have been found between these bunches and the sheathed '9+2' flagella of animals and plants. Vertebrate striated muscle consists of sub-fibrils which are (very roughly) 100 Å thick,

[Quarterly Journal of Microscopical Science, Vol. 94, part 4, pp. 351–367, December 1953.]

250 Å centre to centre and which in resting amphibian muscle have a 400 Å periodicity; in cross sections these sub-fibrils are packed solid (not in hollow cylinders) and in a fairly regular array.

4. Nuclear, cell, and mitochondrial membranes appear double in cross sections (150–300 Å thick); this may be due to the dissolving away of internal lipoid leaving two outer protein sheets, but none of these membranes is thin enough to contain simply a bimolecular lipoid layer. Electron microscope studies of striated cell borders confirm that in some sites there may be distinct filaments of variable length and in others closed ducts, or rods, covered distally by a smooth membrane. One border described contains distinct filaments which join basal mitochondria. It is not yet certain whether the complex internal 'double-membranes' of sectioned mitochondria arise from tubes, or paired sheets, or both.

5. When sectioned after freezing-drying or buffered osmic or formal fixation, the cytoplasm of many protein secreting cells in vertebrates is full of double membranes, like those of mitochondria, and of equally uncertain origin. Sections of many other cells show similar structures, varying in thickness from 75 Å–600 Å. There is some evidence that they are associated with cytoplasmic ribonucleic acid.

INTRODUCTION

It may well be objected that the above title does not embody the proper approach to cytology, which should be by subjects rather than by techniques. It is, however, simply a convenient way of stating that this article will describe several new features of protoplasmic structure at levels of organization below the limit of resolution of the light microscope, and of a kind which either have not been, or cannot conveniently be, studied by any other technique than electron microscopy. And since techniques are so important, they are briefly reviewed in the first section, after which various specialized topics of protoplasmic structure are discussed. Any attempt at completeness either in text or references is impossible within the scope of this article and hence only those subjects and methods are considered which have been investigated in this laboratory during the last few years.

METHODS

Those whose researches have required the routine cutting of 2–3 μ sections by conventional procedures will appreciate the difficulties involved in preparing sections between fifty and one hundred times thinner, as is necessary for high resolution electron microscopy. Fortunately the technical problems have not proved too severe and at the time of writing it is relatively easy to obtain sections about 350 Å thick as shown in figs. 3, 4, 5, and 6, A. These were prepared as follows: (*a*) fix in 1 per cent. osmium tetroxide solution buffered to pH 7·2 with acetate-veronal (Palade, 1951); (*b*) embed in butyl methacrylate (Newman, Borysko, and Swerdlow, 1949); (*c*) section at a microtome setting of 350 Å, using a glass knife (Latta and Hartmann, 1950) and a modified Cambridge rocking microtome identical in principle with the early models manufactured in the late nineteenth century. Techniques of this general kind are now in use in numerous laboratories and though they will doubtless be further improved, they nevertheless represent a substantial dvance over what was possible a few years ago. The great problem is fixation, or minor alterations of the protoplasm, which would be invisible in a light microscope, appear as gross distortions at magnifications of 50,000 to 100,000. There is still only the dimmest knowledge of how and why osmium tetroxide s a good fixative; its special properties have nowhere been more lucidly described than by Baker (1945). They have recently been reinvestigated by Porter and Kallmann (1953). We have found that buffered formaldehyde solutions also make quite good fixatives for the electron microscope level of nvestigation (fig. 6, B, C).

Everything which has so far been published proves that it is impossible to dissolve away the embedding material without destroying the finest details of protoplasmic structure which the electron microscope is capable of revealing (Hillier, 1949). We have made careful comparisons (in ester wax sections) of mouse intestinal brush borders with wax remaining and wax removed which leave no doubt about this point. If the embedding material can be gently volatilized in the electron beam (without fouling the microscope!), well and good (Watson, 1953). Otherwise the presence of embedding medium and the slight consequent loss of contrast must be tolerated, but with adequately thin sections and with tissues whose natural opacity has been enhanced by osmic fixation this loss is small. Dissolving away the embedding medium is perhaps permissible where it is desired only to reveal rather distinct and characteristic inclusion bodies such as plant and animal viruses; and if the embedding medium is to be removed, then still further contrast can be achieved by metal shadowing, as the pictures of Wyckoff and his co-workers show. But the fine structure of the protoplasm in cells with embedding medium dissolved away is so adversely affected that the results are sometimes no better than could be achieved with the light microscope.

Regarding the examination of whole organisms or suspensions of granules or fibres little need be said, as relevant techniques have been discussed by Cosslett (1951). The chief difficulties are: (*a*) the centrifugation and (*b*) the contact with distilled water which may be involved in making clean preparations, and (*c*) the distorting effects of drying (e.g. flattening). All these troubles can be avoided if desired. Clean preparations without contamination can readily be obtained by a draining procedure such as that used by Valentine and Bradfield (1953). Distilled water has little or no harmful effect after proper fixation, which both renders proteins insoluble and destroys membrane semi-permeability. Drying effects can be overcome by freezing-drying, which we have used satisfactorily for several years, or by Anderson's (1950) critical point method. Williams (1953) and Backus (1950) have developed elegant techniques of freezing-drying and spray-drying. But, in fact, the flattening caused by drying is a *sine qua non* for the successful examination of many dense structures (e.g. sperm tails), which without it would be quite opaque at the voltages in current use.

Much criticism hitherto expressed or implied regarding the techniques of biological electron microscopy is without foundation as far as the greater part of the field is concerned. Only in the examination of suspensions of organisms or particles is the drying inherent in electron microscopy a real difficulty and even here there are satisfactory means of overcoming it when desired, as discussed above. For the examination of the vast majority of tissues, by whatever microscope, fixing and sectioning is inevitable, for the size, complexity and dependence on blood-supply of the tissues concerned strictly limit the information which can be obtained from the examination of their living cells (considerable though this sometimes is). For sections, the vacuum desiccation inherent in current electron microscopy constitutes no drawback whatever. Indeed it constitutes an integral part of one of the best methods of preparing tissues for sectioning—the Altmann-Gersh freezing-drying technique. The basic problem becomes the old and ever-present one of fixation, and here the electron microscope procedures, far from being at a disadvantage compared with light microscopy, have much in their favour. The best cytoplasmic chemical fixative known at present is a buffered solution of osmium tetroxide, but this is very little used in light microscopy because it often leaves tissues in a crumbly condition difficult to section, and in an altered chemical condition difficult to stain. Neither of these tendencies is detrimental in current electron microscope procedures (as far as cytology, as distinct from cyto-chemistry, is concerned), and indeed the osmium is an excellent electron stain. Moreover the electron microscope procedures involve only fixation, embedding, and sectioning; apart from examination in the microscope itself, at normal low beam intensities, there is no further treatment after sectioning. They thus avoid all the stages of de-waxing, hydration, staining, and dehydration in which artifacts may be introduced during the course of normal cytological practice for the light microscope.

The following is a summary of the techniques used in making the preparations illustrated in the plates.

Fixation. Figs. 1, A, E, and 2, A–C: 2 per cent. formaldehyde solution. Figs. 1, B–D; 2 D, E; 3; 4; and 5: 1 per cent. osmium tetroxide solution buffered to pH 7·2–7·4 with acetate-veronal (Palade, 1951). Fig. 6, B, C: 2 per cent formaldehyde solution similarly buffered.

Sectioning. Figs. 1, A–D; 2, D, E; 3; 4; 5; and 6 A: butyl methacrylate at a microtome setting of 0·035μ. Fig. 6, B, C: ester wax at 0·14μ. (All with embedding medium undissolved.)

Shadowing. Figs. 1, E and 2, A–C: 60 per cent. gold and 40 per cent. palladium alloy at approximately 30° to the horizontal.

Microscopy. Siemens electron microscope at 70 kV.

Fibrillar Contractile Structures—Flagella 1–5 and Muscle

Flagella. It has long been known that animal and plant flagella contain fibrillar protein oriented parallel to their long axes, but the detailed arrangement of the fibrils is only just being elucidated. In the case of bacteria, the resolution limit of the light microscope is such that there has even been controersy (reviewed by Knaysi, 1951) over the existence of bacterial flagella. The ontributions of electron microscopy in this field may be summarized thus:

1. Number of fibrils. All animal and plant flagella which have so far been xamined in detail contain a definite and relatively small number of component fibrils which are between 100 Å and 200 Å thick. In many cases (cock perm, Grigg and Hodge (1949); Ulothrix, Fucus and Chaetomorpha sperms, *Paramecium*, an unidentified mollusc, frog, human ciliated epithelium—references in Manton (1953); cilia of *Mytilus edulis* (personal observation); sperms of several species of slug and one species of cockroach (Cameron) and of *Paracentrotus lividus* (fig. 1, A), it is clear that the total number of component ibrils is 11—an outer ring of 9 similar fibrils and 2 other fibrils, probably thinner and centrally placed and certainly different chemically from the other 9. In particular the 2 fibrils are more sensitive to distilled water and digestive enzymes, so that it is all too common to find only 9 fibrils remaining in a macerated flagellum where there should in fact be 11. It can be claimed that any number just below 11 should be regarded as false owing to loss of fibres, but the admittedly widespread distribution of the 9+2 pattern must not be allowed to blind us to the possibility of other patterns. Indeed, it has recently become clear that a more complex pattern does exist in some, possibly all, mammalian sperm mid-pieces, where there are *two* concentric cylinders of nine fibrils plus the usual central pair—20 in all (fig. 1, B–D; Challice, 1953; Bradfield, 1953). The fibrils of the inner cylinder are more slender than those of the outer cylinder and of about the same thickness as the central pair. The exact doubling of the peripheral fibrils only serves to emphasize the significance of the number 9, which is at present a mystery. It is, however, noteworthy that in bull and ram sperm, for instance, the fibrils are gathered into 3 bundles in the neck region, each connecting with one of the 3 granules set in the base of the head. It is not known whether the bundles are similarly constituted, but at least the number of peripheral fibrils (9+9) is exactly divisible by the number of bundles (3)—as if there were 3 primary contractile units, each subdivided to facilitate finer gradation of effort. Three such units would presumably be the minimum apparatus necessary to produce 3-dimensional waves (whether these generate a circle or a very flattened ellipse when viewed end-on) in a sperm tail consisting of straight contractile fibrils. The chemical nature of the tail helix (see below) argues against its being contractile, though this point has not been finally settled yet.

Frey-Wyssling's (1953) recent survey of sperm tail structure is unfortunately misleading. The statements regarding 8 fibrils in a tube around one central fibril (presumably for bull sperm, though this is not specifically stated), 6+6 for ram sperm, and 9 for sea-urchin sperm are all incorrect (see figs. 1, A–D for two of these sperm).

The most complicated flagella apart from mammalian sperm-tails are probably the so-called flimmer-flagella found in numerous Protista and in some cryptogam sperm. Despite studies by Houwink (*Euglena*), Manton (*Fucus*), and in this laboratory (*Ochromonas*), the detailed relation of the fine lateral branches to the main axial fibres is not fully understood.

Although there may be chemical differences between them, the component fibrils of a flagellum usually have the same length, within a few mμ. Notable exceptions are found among fern sperm (fig. 1, E) where an elegantly tapered point results from the way in which the component fibrils end one by one until only a single fibril (or triplet?) runs to the extreme tip.

2. Sheaths and membranes. In all multi-fibrillar flagella which have been studied there are one or more sheathing membranes binding the fibrils together. This is particularly conspicuous in the fern sperm flagella mentioned above (fig. 1, E), where the sheath commonly becomes displaced in a peculiar and characteristic manner. Round mammalian sperm tails there is, in addition to a sheathing membrane, a stout double helix, composed at least partly of fibrillar protein, which we have found to be strongly resistant to 0·2N hydrochloric acid and 6M urea, but dissolved by 0·1N sodium hydroxide. This cortical helix occurs in all mammalian sperm studied up till now, including a marsupial sperm of which we have taken a few photographs (fig. 2, A), and has not been found outside Mammalia. It may therefore constitute one of their diagnostic characters.

3. Bacterial flagella. A bacterial flagellum consists of a single fibril of very even diameter, closely similar in size to one of the component fibrils of multi-fibrillar flagella, often originating from a basal granule and very distinct from a strand of mucus. No such flagella have been detected in viruses or *Rickettsiae*, so that this single fibril is presumably the basic evolutionary unit in the flagellar hierarchy. It displays just as wide an evolutionary radiation as do the more complex multi-fibrillar flagella. There may be a single polar flagellum, or one at each pole; or numerous flagella regularly arranged over the bacterial surface; or a bunch of flagella at one pole or at both poles (*Spirilla*). Or, finally, instead of hanging free, the fibrils (one or many) may be wound spirally round the elongated axis of the body (fig. 2, C) and attached at both ends: this is the interesting structural device which distinguishes the spirochaetes (Bradfield and Cater, 1952), there being only 1 fibril in the smallest (*Leptospirae*), from 4 to about 30 in genera of intermediate size (*Treponema* and *Borrelia*), and a bundle of more than a hundred in the larger species such as *Cristispira*.

FIG. 1 (plate). A, Sea-urchin sperm. T. S. showing ring of 9 fibrils surrounding 2 thinner central fibrils. × 40,000. B, Bull sperm. Slightly oblique T. S. of mid-piece showing outer mitochondrial sheath, inside this a cylinder of 9 thicker fibrils (showing here as short rods on account of the oblique cut), inside them a cylinder of 9 thinner fibrils, and inside them a pair of fibrils very close to each other and similar in thickness to the fibrils of the inner cylinder (see D). × 45,000. C, Bull sperm. T. S. of tail distal to mid-piece, showing a ring of 9 fibrils around a central pair; there may be an intermediate ring of very fine fibrils, or the 9 conspicuous fibrils may be double, this is not certain yet. × 72,000. D, Bull sperm. Diagrammatic clarification of the *inner* 9+2 fibrils in B; suggested 3-dimensional aspect is given by widening the fibrils on one side. E, Bracken sperm, tip of flagellum. Component fibrils end one by one. The ends of the 9 main fibrils are indicated by arrows. Sheathing membrane artificially displaced. × 70,500.

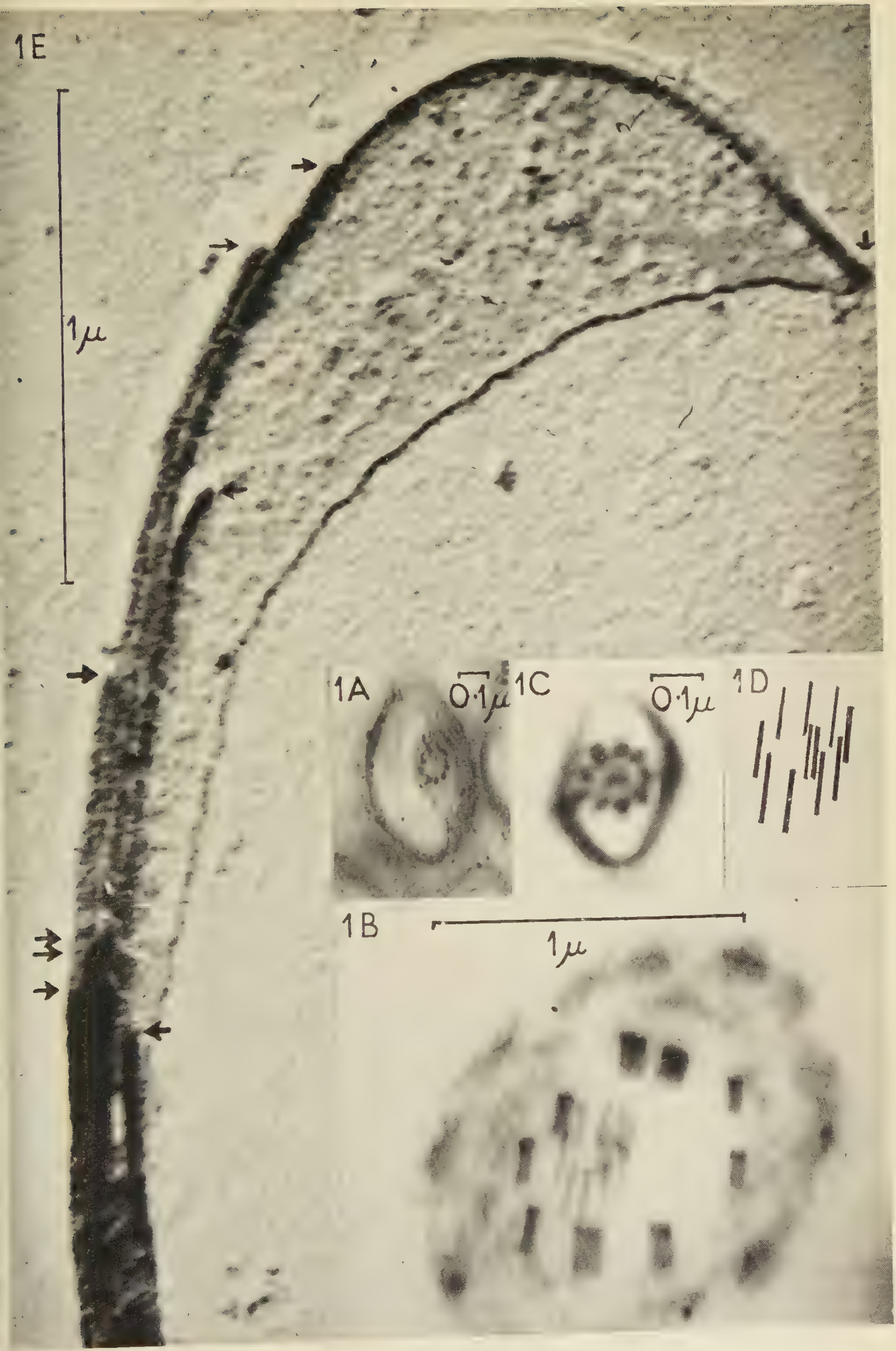

Fig. 1

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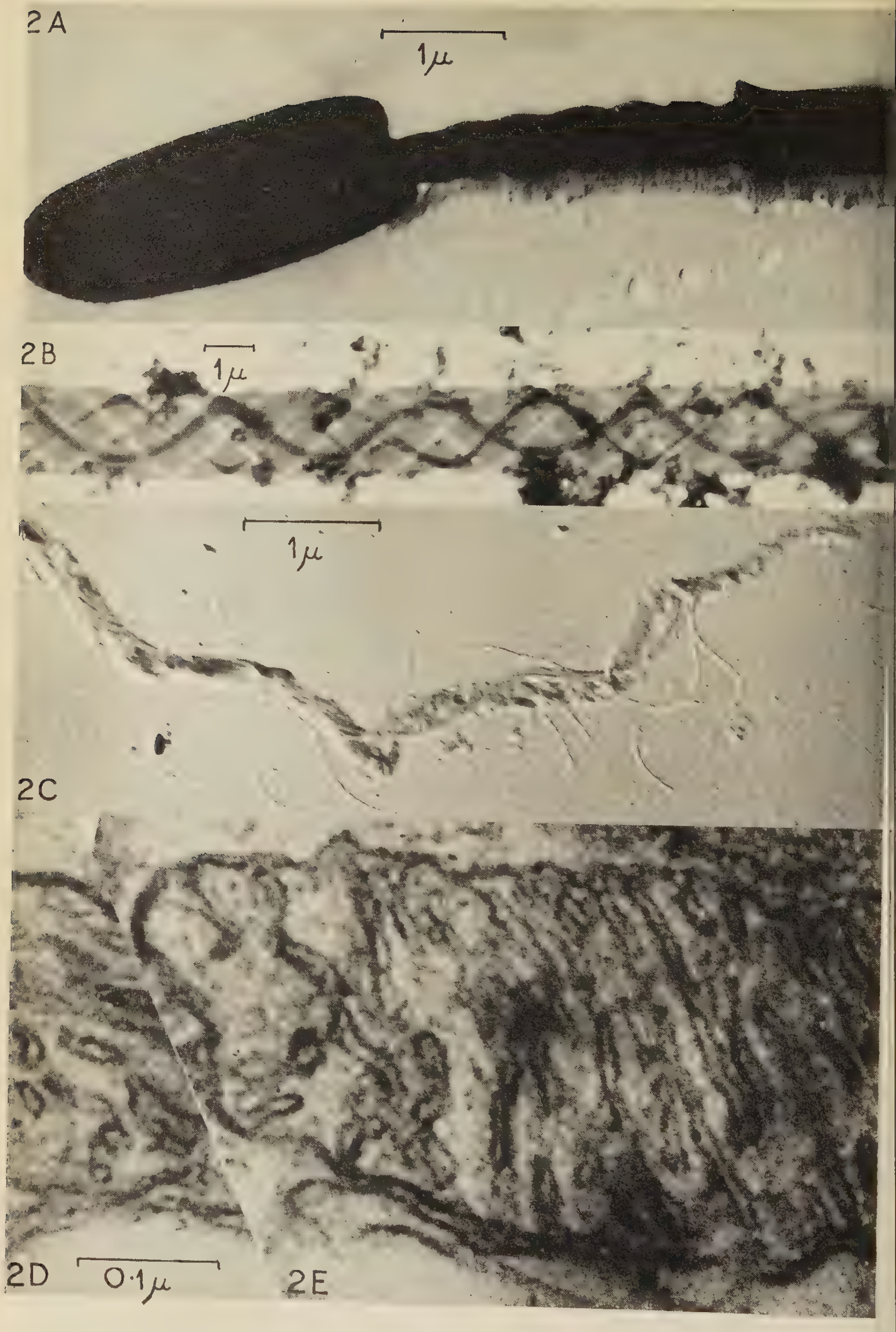

Fig. 2

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But neither in the large spirochaetes nor in the *Spirilla* has any trace been found of a sheathing membrane surrounding the bundle or bunch of fibrils. In fact no intermediates whatever have yet been found between the collection of separate bacterial fibrils and the true multifibrillar flagellum with a well-defined membrane sheathing its 11 or more fibrils. It is hardly credible that this complex arose at a single step. Intermediates may of course have become extinct, but it will be interesting to hunt for them among the vast assemblage of present day Protista.

4. Mode of action. If we assume that the ring of fibrils in a multi-fibrillar flagellum are contractile, it becomes relatively easy to make hypotheses about the behaviour of the flagellum (or cilium) as a whole (Gray 1928), whether 2- or 3-dimensional. Plenty of physiological problems remain, and why there should be the odd combination of 9+2 is likely to be a puzzle for some time. But it is necessary only to postulate the power of linear shortening in the component fibrils of the ring in order to make simple hypotheses of movement.

On the other hand, it is by no means clear, in the case of a uni-flagellate bacterium, how waves could be propagated down a single fibril 100–200 Å in diameter, equivalent to one component fibril of the multi-fibrillar flagellum discussed above. It may be that the motif is repeated on a smaller scale and that each 100–200 Å fibril itself consists of a bundle of smaller fibrils in the manner of Dean Swift's fleas; but the process could certainly not go on *ad infinitum*, for the sub-fibrils within a 100–200 Å fibril would already be of the order of diameters of single protein molecules. The alternative (apart from passive waggling by a structure active at the base of the flagellum) requires the generation of waves by contraction within a large, single 'molecule' or string of molecules—either by local contractions passing down opposite sides out of phase (2-dimensional waves), or by local contractions passing round a helical structure such as is now favoured for some protein molecules (3-dimensional waves). The recent suggestion by de Robertis and Franchi (1951) that some bacterial flagella consist of a helical core inside a distinct sheath would provide a possible structural basis for the latter idea. We have not observed a similar structure in the bacterial flagella studied here, but this may be due to faulty technique or unsuitable species.

5. Non-fibrillar flagellum-like structures. In conclusion it is of interest to refer briefly to the remarkable filamentous appendages of Crustacean sperm, which look as if they might be constructed on the flagellum-pattern but are

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FIG. 2 (plate). A, Opossum sperm. Helix round tail, which is the paler structure underlying the head and mid-piece of another sperm, the latter being too dense to show structure. (These sperm were in poor condition when obtained.) × 17,500. B, *Notodromas* (Ostracoda) sperm. Empty tail sheath showing imprint of the 6 helical components originally inside (4 broad and 2 narrow). × 7,000. C, *Treponema duttoni* (African tick fever spirochaete, cultured in mice), after mild trypsin digestion which slightly disrupts (thereby displaying) the bundle of fibrils which is wound helically round the organism and can be seen passing alternately over and under the body in this shadowed preparation. When intact, the fibril-bundle is tightly wound round the surface of the organism. × 19,500. D and E, *Chironomus* salivary gland; detail of mitochondria (see fig. 3). × 205,000.

not. These sperm are said to be without true flagella and we have confirmed that in the three appendages of lobster sperm, for example, there is no sign of fibrillar structure of the kind or on the scale found in true flagella (Rothschild, 1952). Equally unusual are the tails (called heads by Retzius) of freshwater Ostracod sperm, where the customary fibre-bundle plan has been abandoned in favour of an extraordinary 6-thread spiral flex (fig. 2, B). Other Crustacean and some Arachnid sperms are equally aberrant in this respect, but recent electron microscope studies in this laboratory show that at least some insect sperms are very close to the 'archetype' tail structure (Cameron).

Muscle. Electron microscopical additions to our knowledge of the fine structure of striated muscle may be summarized thus:

1. Myofibrils can be resolved into fine filaments about 100 Å wide and showing a marked 400 Å axial periodicity.

2. Myofibrils are packed solid with these fine filaments disposed in regular array (Morgan and others, 1950), about 250 Å centre to centre, and are not hollow cylinders with walls one filament thick as was once thought possible. This was first demonstrated by Morgan and others (1950), and has been confirmed in this laboratory and by several other workers, particularly Huxley.

3. Draper and Hodge (1950) state that in toad muscle the axial periodicity in the filaments varies from 400 Å–250 Å, roughly in proportion to the sarcomere length and degree of contraction. They are tentative about this, however, and about differences in periodicity in different bands of a sarcomere, and there are obviously several important questions needing further investigation here.

NUCLEAR, MITOCHONDRIAL, AND CELL MEMBRANES, AND 'BRUSH BORDERS'

The outstanding feature about this group of membranes is that each appears to consist not of a single layer of its constituent molecules, but of at least two distinct layers. To a certain extent this may simply be a direct confirmation (and extension to nuclear and mitochondrial membranes) of the double lipo-protein layer theory of cell membranes described by Danielli (1941), as Sjöstrand (1953 *a*, *c*) has suggested—the central lipoid being lost during the preparation of sections and the two outer protein layers remaining to produce the double structure observed. But the principle extends to cell walls which are not primarily lipo-protein in nature, as in the very interesting yeast cell wall investigated cytochemically by Northcote and Horne (1950). Here it appears that one layer consists mainly of a glucan and the other of a mannan plus lipo-protein (a varied mixture!). Probably the latter will be subdivided by future work.

Electron microscopy has so far shed little light on the internal structure of the nucleus, for a number of reasons which need not be elaborated here. The nuclear membranes of two amphibian oocytes have, however, been elegantly investigated by Callan and Tomlin (1951), who find each to consist of an outer, porous membrane (pores 400 Å diameter and 1,000 Å centre to centre) and an inner continuous membrane; both membranes contain relatively insoluble protein and the outer in addition contains some lipoid. Double nuclear membranes have now been found (in sections) in such diverse sites as mammalian kidney and liver, insect silk glands and amoebae, but it remains to be seen whether these resemble chemically the oocyte nuclear membranes.

There has long been evidence for the existence of membranes round mitochondria. But even in 0·2 μ sections, mitochondria are too dense to reveal details of their structure. Only since it has become feasible to cut sections of the order of 0·05 μ, or less, has it been possible to observe the detailed structure of sectioned mitochondria. Sjöstrand's micrographs show that the mitochondria of mouse kidney proximal convoluted tubules are bounded by a double membrane 160 Å thick, the two component laminae being 45 Å thick and 115 Å centre to centre. In addition these mitochondria contained 'a system of internal double membranes oriented chiefly transversely to the long axis of the mitochondria'. Sjöstrand (1953*c*) states that at their outer edges the two constituent laminae of the inner double membranes are joined so that the space inside them is closed, but in other respects the inner and outer membranes are identical. In other words his mitochondrion resembles a double-walled money-box containing a stack of hollow coins (not necessarily circular).

This may well be the case in many mitochondria, but in various insect cells (salivary glands of *Chironomus* and Malpighian tubules of *Galleria*) which we have studied it appears likely that at least some of the internal double membranes of mitochondria arise from tubules and not from hollow disks or sheets. Mitochondria were not our primary object of study, but they are very conspicuous in these cells and in both sites the sectioned mitochondria showed (in addition to longer runs of double membrane) numerous small circles and ellipses (figs. 2, D, E; 3; 4). Clearly the latter may be tubes in cross section, but they may also be granules in cross section, and here only serial sectioning can decide. Both this and the important question of fixation artifacts will be discussed in the next section, but it is worth noting here that few structures could be better placed for fixation than the mitochondria in figs. 2, D, E and 3, which are only 2–3 μ from the outer cell border and hence from the fixative. Even slowly penetrating osmium tetroxide must reach them very soon after the cells come into contact with fixative, which in this case was immediately the animal was killed by decapitation.

Electron micrographs of the outer ends of retinal rods (Sjöstrand, 1949) and of chloroplasts (Leyon, 1953) show that both contain stacks of discoid lamellae. It is hard to believe that chloroplasts are, as stated by Leyon, devoid of outer sheathing membranes.

In contrast to the mitochondrial membrane whose existence, though postulated, was far from certain before it was identified in the electron microscope, the cell membrane, about which so much has been written, is often quite difficult to observe in electron microscope sections. Sjöstrand (1953*c*) states that in the proximal convoluted tubules of mouse kidney, the cell membranes consist of two 60 Å laminae (presumably protein) separated by 160 Å. The latter is too great to be spanned by a bi-molecular layer of lipoid.

In some highly differentiated cells, the boundary membranes undergo elaboration at a level of organization where the electron microscope can reveal useful new or confirmatory information. Thus in the myelin sheath of axons, the micrographs of Fernández-Morán (1950) and Sjöstrand (1953*b*) have given direct confirmation (in general arrangement if not in detailed dimensions) of the multilayer structure predicted from X-ray diffraction. With regard to the interesting brush-borders which are common in absorptive epithelia, the rod versus pore controversy, dating from the mid-nineteenth century, rages as fiercely as ever (J. R. Baker, 1942; Granger and R. F. Baker, 1950). As Wigglesworth (1950) points out, it is very likely that both are true, in different cells. From comparisons of sections with and without embedding medium, we are certain that removal of the latter seriously disrupts such fine structures as these, and this factor may well account for the distinctly separate rods demonstrated by Granger and Baker (1950), Bretschneider (1952), and others. Sjöstrand (1953*c*) believes that the brush-border of mouse kidney proximal convoluted tubules consists of fine closely packed ducts, circular in cross section, filled with protoplasm, closed at each end, with ramifying tubes leading down into the cytoplasm from their inner ends, and covered by a continuous smooth membrane at their outer (distal) ends next to the lumen. On this view the spaces between the ducts do not communicate with the lumen of the kidney tubules and Sjöstrand does not therefore subscribe to the view that they are a means of increasing absorptive area.

It seems that a straight, closed 'duct', filled with protoplasm, might more simply be called a rod, but what matters is not nomenclature, but the thorough understanding of the structure, which Sjöstrand rightly describes as very intricate. We have studied the striking borders of insect Malpighian tubules, particularly in the waxmoth caterpillar (*Galleria*). It is very probable that, as Wigglesworth (1950) has shown for *Rhodnius*, there is a honeycomb border in the upper region of these Malpighian tubules and a true brush-border in the lower regions. Our sections are from the lower regions and have been examined without removal of methacrylate. There seems no doubt that, as Wigglesworth found for the corresponding zone in *Rhodnius*, the border consists of separate rods of variable length. It is unlikely therefore that they can be covered with a continuous outer sheet, and none has so far been seen. But more interesting than the outer border is the inner (proximal) end of each rod, which connects in many cases with a typical mitochondrion; a few clear examples of a junction between brush-border rod and underlying mitochondrion, as well as several 'near-misses', may be seen in fig. 4. Some of the mitochondria in question are full of the double membranes discussed above; others contain numerous

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FIG. 3 (plate). *Chironomus* salivary gland. L. S. outer 3–4 μ of cytoplasm which is rich in tracheoles (empty circles) and mitochondria. The latter show conspicuous 'double membranes' (sometimes transverse, sometimes longitudinal, often branched), but also contain irregular circles or ellipses, which may be tubes or granules in T. S. (See Fig. 2, D, E.) × 75,000.

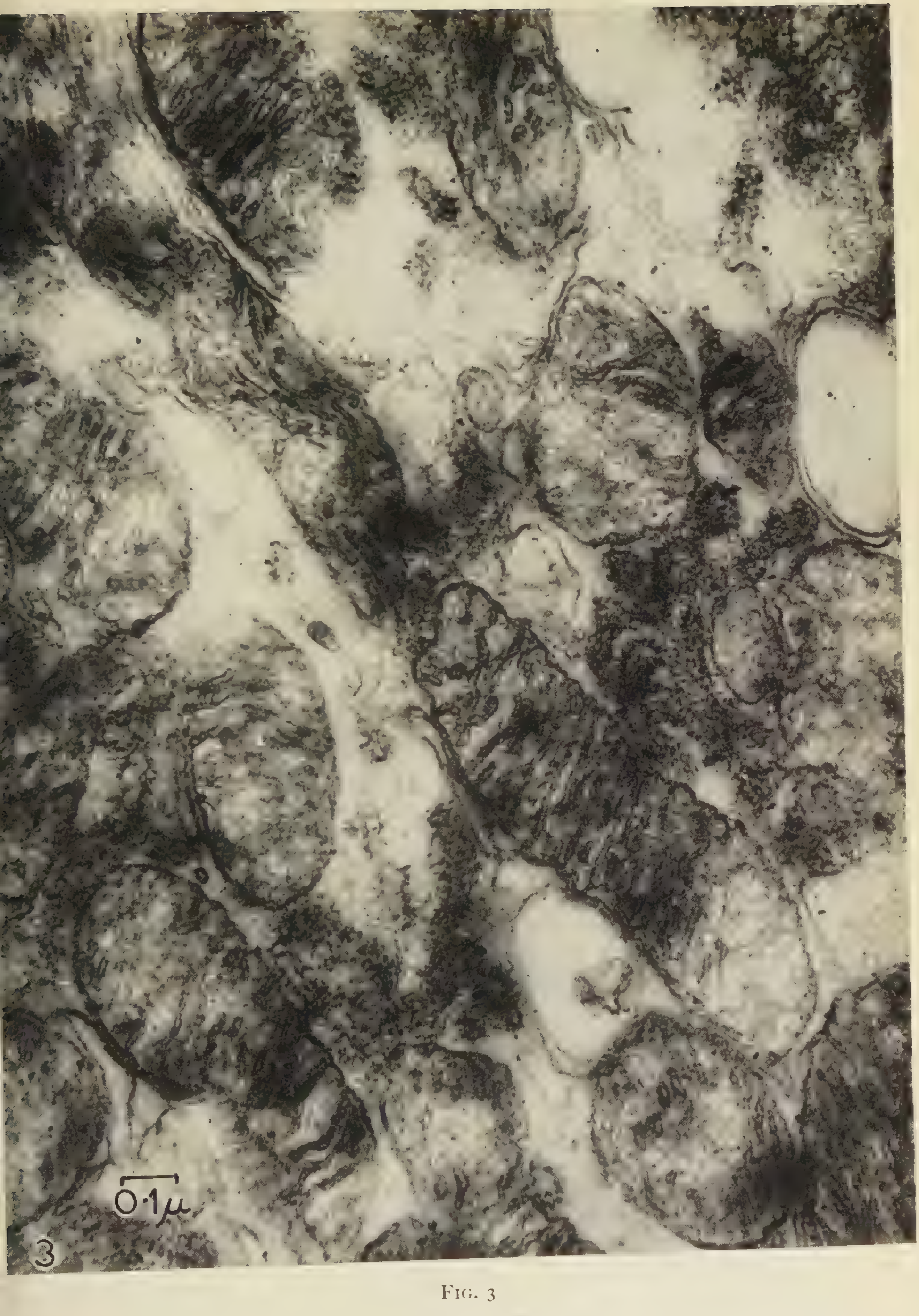

FIG. 3

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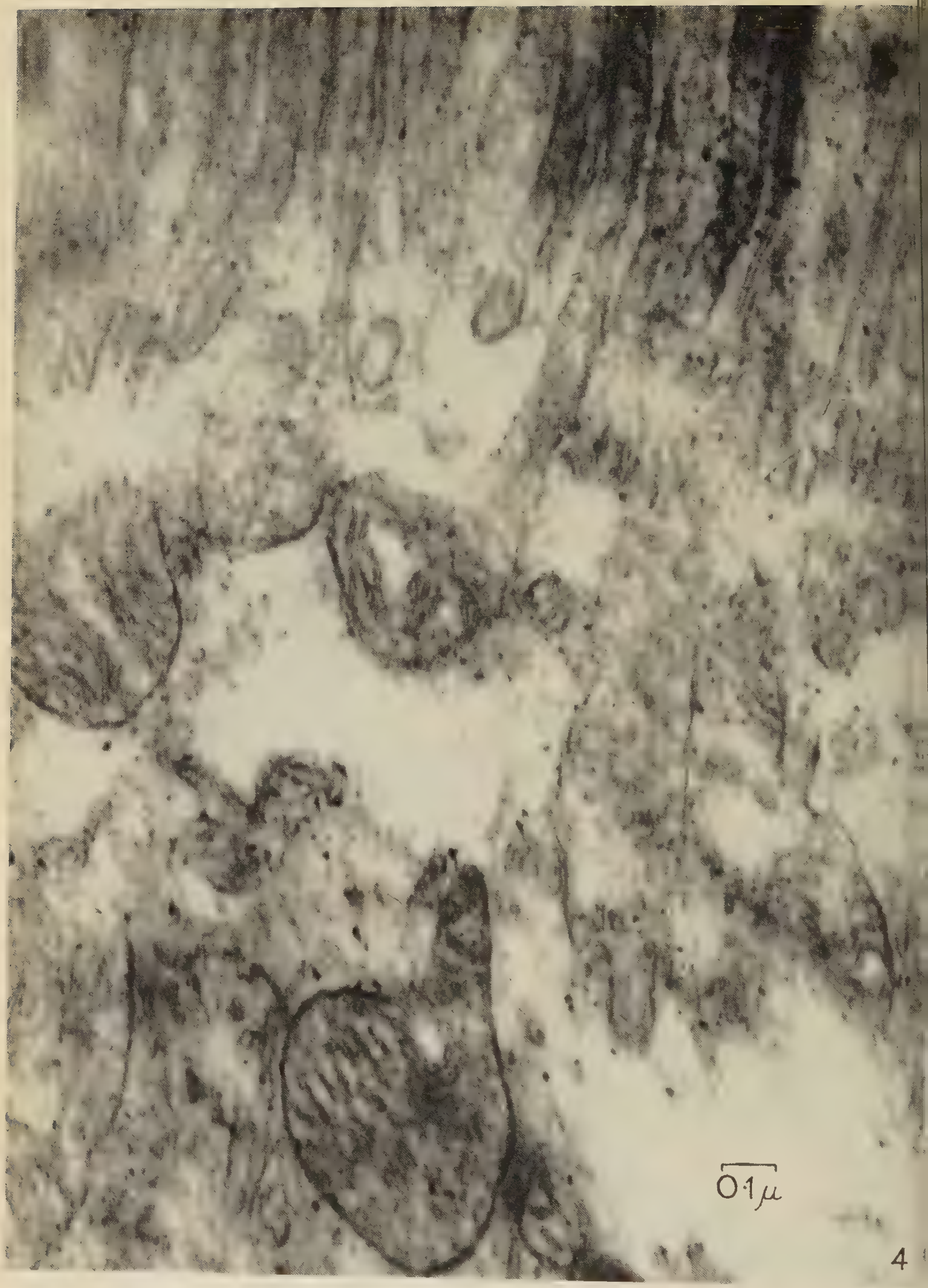

FIG. 4

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regular circles and ellipses which are probably oblique transverse sections hrough tubes (though they might of course be granules, and only serial ections can decide). Whether they prove to be derived from laminae or ubules, it is noteworthy that the double membranes extend from the basal nitochondria up into the brush-border rods, thus emphasizing the similarity of the latter to mitochondrial extensions or appendages. These basal mitochondria probably correspond to the 'basal granules' which have long been nown and which are picked out by mitochondrial stains (J. R. Baker, 1942).

We are of course using the characteristic internal structure which has just een shown to exist in mitochondria as a criterion for identifying new granules as mitochondria. In fact, this should be only one of several criteria, including vital staining and enzymological characteristics (reviewed in Bradfield, 1950) used to define these bodies, and with such diverse criteria available it seems time for a comparative study of whether all such features always exist side by side, or whether various combinations and gradations of properties occur among the great array of mitochondria-like cytoplasmic particles found in living cells.

Cytoplasmic 'Double Membranes'

Among the most interesting features of protoplasmic structure so far revealed by electron microscope study of thin sections are the so-called cytoplasmic double membranes (figs. 5; 6, B, C). They appeared at first as long fibrils (Hillier, 1949; Dalton and others, 1950), but were later found to be double and resolved into pairs of parallel lines (Bernhard and others, 1952; Sjöstrand, 1953*a*) and hence their current name. It is already evident that the total thickness of double membranes may vary considerably from tissue to tissue. For mouse pancreas the figure is approximately 600 Å, or 360 Å centre to centre (fig. 6, B, C); for mouse kidney proximal convoluted tubules 270 Å, or 190 Å centre to centre (Sjöstrand, 1953 *a*, *c*)—though until it is proved that these structures are sheets and not tubes (see below) it is hardly admissible to take an unbiased average thickness; and for waxmoth caterpillar Malpighian tubules the total thickness may be as low as 75 Å, or 50 Å centre to centre (fig. 5). In the few tissues already studied there is, therefore, almost a tenfold variation in total thickness and it is questionable whether all double membranes, wide and narrow alike, have the same kind of organization. The thinnest which we have encountered—in caterpillar Malpighian tubules—are of the same order of thickness as the intramitochondrial double membranes in the same cells and *thinner* than the intramitochondrial double membranes of some other cells (mouse kidney and *Chironomus* salivary glands). In some protein-secreting cells such as those of mouse pancreas (Sjöstrand, 1953*a*, and

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FIG. 4 (plate). Malpighian tubule of the caterpillar of the waxmoth (*Galleria*). L. S. showing small area at inner (proximal) end of the brush-border filaments. A few filaments are seen to arise directly from basal mitochondria and the 'double membranes' inside the latter extend up into their respective filaments. Some mitochondria contain circles and ellipses suggestive of tubes or granules in T. S. ×70,000.

fig. 6, B, C) and rat salivary gland there are such serried arrays of double membranes that the sectioned cytoplasm looks like a miniature railway marshalling yard. In other cells, such as mammalian kidney, liver, and intestine and insec Malpighian tubules, the double membranes are present but less numerous In mammalian neurones Hagenau and Bernhard (1953) find nothing quit like the double membranes of kidney or pancreas and that has been ou experience with *Chironomus* salivary glands and caterpillar silk glands, whic is rather surprising in view of the fact that mammalian protein-secreting cell possess abundant double membranes. However, our provisional interpretatio of *Chironomus* salivary glands (fig. 6, A) is that the bulk of the cytoplasm consists of a tangled mass of ramifying tubules 500–600 Å in external diameter Because of their convolutions none of the tubes runs very far in any one sectio and hence they do not give the same visual impression as 'double membranes' but if the latter turn out to be tubes rather than sheets (see below) the two structures may well prove to be of the same general kind.

As indicated above, these discoveries raise several problems. Do the double membranes seen in sections arise from paired sheets, or are they chance longitudinal sections of tubes? And does the parent structure in the embedded tissue exist as such in the living cells, or is it an artifact? (Even if it were, the observations cannot be dismissed as meaningless, for such intricate structure must reflect pre-existing complexity—either chemical or structural.)

With regard to the possibility of fixation artifact, it is noteworthy that very similar double membranes have been observed in exocrine pancreas cells fixed in buffered osmium tetroxide solutions, or by freezing-drying (Sjöstrand, 1953*a*), and in buffered formaldehyde solutions (fig. 6, B, C). The only differences are in minor details of spacing and thickness. The fact that two good (but very different) chemical fixatives, together with an excellent physical method of fixation, all give closely similar results provides strong evidence that the parent structure does exist in living cells. Structures of this kind naturally bring to mind the danger of myelin-figure formation. Palade and Claude (1949) have shown this danger to be serious when a wave of acidification advances through the tissue, favouring the formation from some phospholipoids of myelin figures, which are then fixed by the succeeding wave of more slowly penetrating osmium tetroxide. The acetate-veronal buffered fixative recommended by Palade (1951) and used in most recent electron microscope studies should minimize the formation of myelin figures, and during freezing-drying conditions are also unfavourable for their formation. To settle the reality of these structures by direct observation in living cells is unfortunately impossible in view of their size. But extensive arrays of them might well give rise: (*a*) to cytoplasmic striations visible in the light microscope; (*b*) in special cases to birefringence (Sjöstrand, 1953*a*); (*c*) to canals of the kind convincingly

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FIG. 5 (plate). Malpighian tubule of the caterpillar of the waxmoth (*Galleria*). Similar to fig. 4, but the section is of a region near a nucleus (indicated by arrows in the bottom right-hand corner) where the mitochondria are smaller and the cytoplasm contains very narrow double membranes only 75 Å thick (50 Å centre to centre). × 95,000.

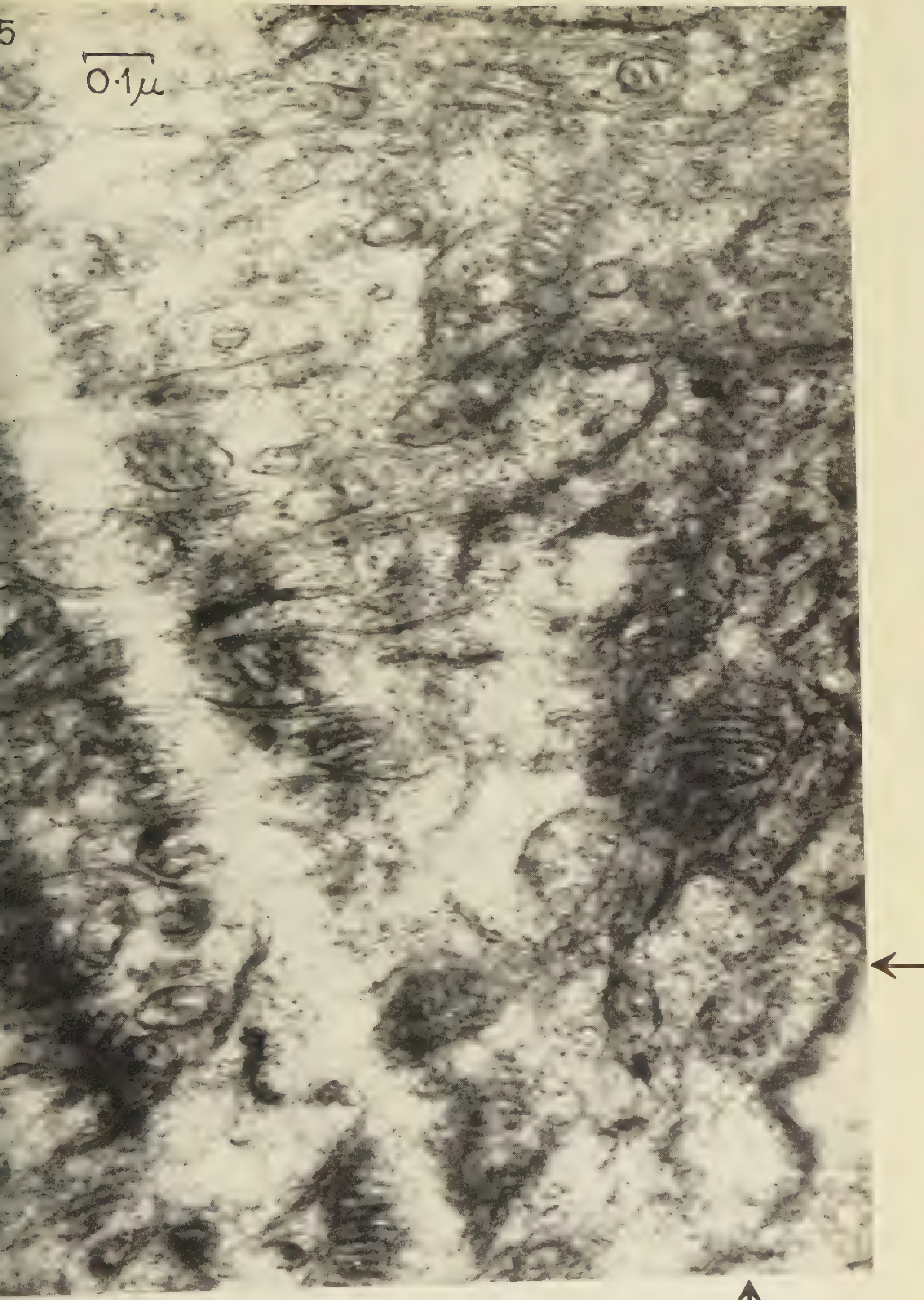

Fig. 5

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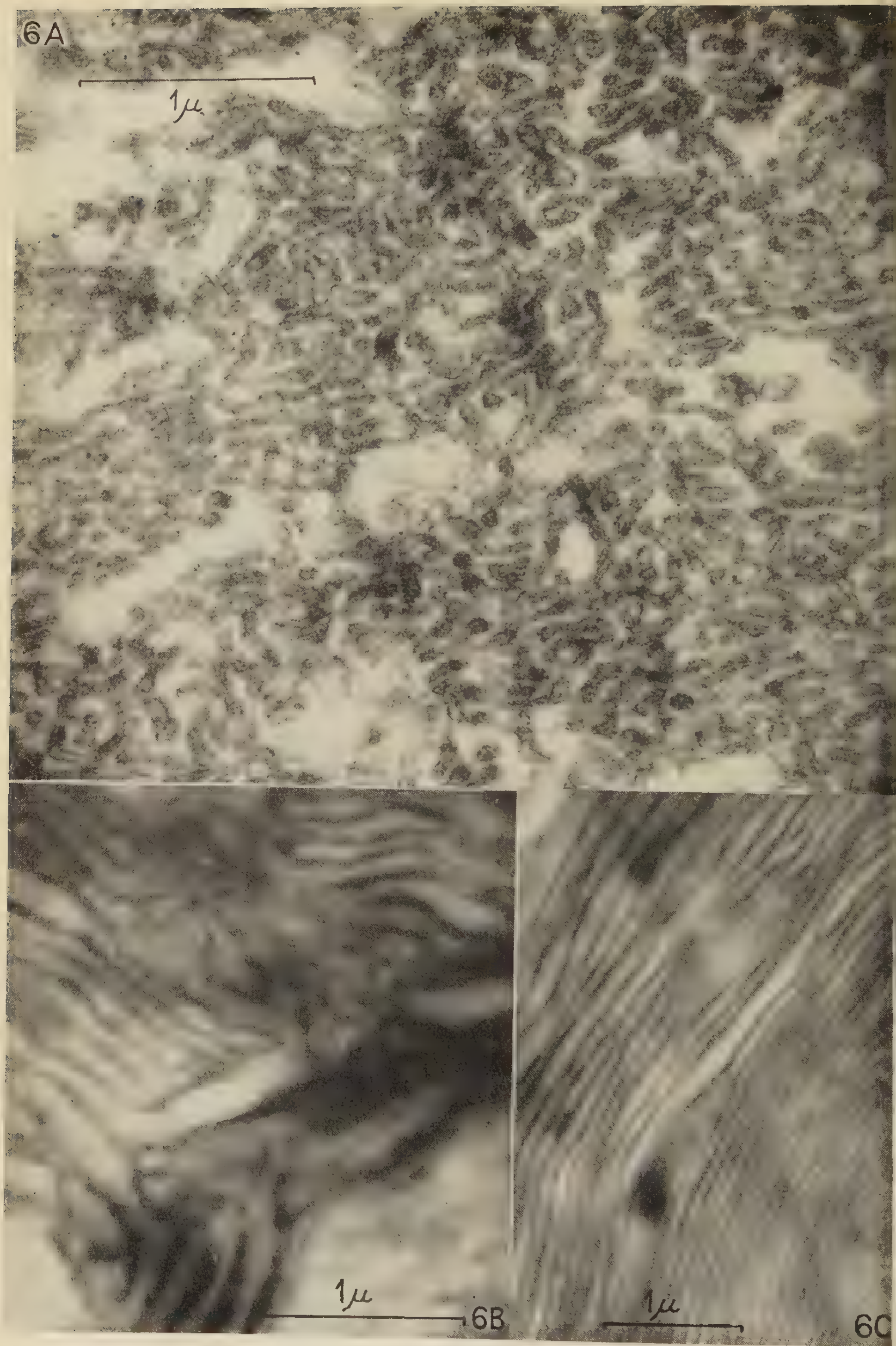

Fig. 6

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lemonstrated recently by Ludford and Smiles (1953) in living polymorphic arcoma cells, or of the kind reviewed by Bensley (1951) in his clear reassertion f the view that living cells contain complex arrays of canals and vacuoles imilar in general, though not in detail, to the complex artifacts produced by lassical Golgi methods. The parent structures of the double membranes bserved in sections may well have been concerned in the production of lassical Golgi pictures, but when the tube versus sheet debate is settled, it vill undoubtedly be better to give them a name based on their form (in the nanner of 'mitochondria') rather than to regard them as the new manifestation f the Golgi complex, for the latter term has outlived its usefulness.

With regard to the form of the structures which give rise to the double nembranes seen in sections, no definite answer can yet be given. At first they vere thought of only as fibrils (Hillier, 1949; Dalton and others, 1950), but ater Dalton (1951) took the view that they were lamellae and Sjöstrand is of the same opinion. 'It is quite obvious that we are dealing with membranes and not filaments from the fact that they may be followed without interruptions through the whole basal cell zone, the chance to hit a filament so exactly along its entire length being negligible. In addition there have never been any indications of cross-cut filaments' (Sjöstrand, 1953*c*). The latter sentence is not true for our sections of mouse pancreas and *Chironomus* salivary glands (fig. 6, A), where there are plenty of indications of tubes cut in cross section; but these are only *indications*, for irregular circles and ellipses could be transverse sections either of tubes or of granules, and only serial sections can settle the matter. The validity of the first sentence is very hard to assess. Sjöstrand does not state the thickness of his excellent sections, but they appear to be of the same order of thickness as those shown in figs. 2, D, E, 3, 4, 5, 6, A, which were cut at a microtome setting of 350 Å. The probability of the parent structures being tubes, therefore, depends on the chance of getting a stretch several μ long of a 270 Å thick tubule in a 350 Å section through a cell containing an unknown number of such tubules—which may be oriented randomly or in a preferred direction. Again depending on the thickness of the section, paired sheets would not always generate the sharp double lines actually observed; the latter would appear only when the section cut the sheets at approximately a right angle (not more than 15°–20° either way). Obviously the relative probabilities are hard to calculate and it is better to defer judgement until the matter is determined by serial sectioning.

Porter (1953) has described in isolated tissue-culture cells the 'endoplasmic reticulum', which consists of irregular canals 500–1,000 Å wide, or in the case of vesicular units up to 6,000 Å wide. These structures may well belong to the same general family as those which give rise to the well-defined double mem-

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FIG. 6 (plate). A, *Chironomus* salivary gland. Similar to fig. 3, but the section is of a region near the cell centre which could be interpreted as a mass of tangled tubules of about the same thickness (600 Å) as the 'double membranes' in, for instance, mouse pancreas. × 33,500. B and C, Mouse pancreas (formalin fixed), showing cytoplasmic 'double membranes' at different magnifications. B, × 29,000. C, × 19,000.

branes. But we cannot agree that the latter are 'easily identified' with Porter endoplasmic reticulum in cultured cells (and have not been able to verify th reference to a paper by Palade and Porter (1952) where this claim is said t be substantiated). On the contrary, there are profound differences in degre if not in kind, between the two types of structure. First, the endoplasmi reticulum is extremely variable in diameter in a single strand, and in a singl cell type, with a *lower* limit of 500 Å (this could be partly due to flattening i the remarkably thin cells from which Porter obtains his excellent micrographs whereas the double membranes are fairly regular in diameter in a single ce type with an *upper* limit, so far, of about 600 Å (mouse pancreas)—some bein apparently as thin as 75 Å (fig. 5). Second, the endoplasmic reticulum i abundant in cultured cells of kinds (e.g. endothelial cells) where one would no expect to find abundant double membranes, for the latter are not a majo cytoplasmic constituent in most cell types so far studied.

Only in sections of cells noted for their abundant protein secretion ar double membranes known to be anything like as abundant as is the endoplasmic reticulum in almost all the cell types described by Porter.

If the double membranes seen in sections are homologous with anything i Porter's cultured cells it is probably with the dense filaments of growing cell (his figs. 5 and 19), which because of their supposed relation to 'growt granules' are labelled 'gg'. Porter makes the interesting observation that thes filaments have a considerable intrinsic density, independent of osmic fixation which is exactly our experience with pancreatic double membranes, which ar almost as dense after fixation in formaldehyde as after osmic fixation.

Little can at present be said about the constitution and function of doubl membranes, or about their formation and growth—unless they do in fac correspond to Porter's dense filaments (see above), which he believes to be formed by the aggregation of 'growth granules'. Sjöstrand suggests tha double membranes, like cell and intramitochondrial membranes, may be composed of an inner layer of lipoid, removed during preparation, and an outer coating of protein. This view is quite plausible, but there is no direct evidence for it yet. The thin double membranes seen for instance in fig. 5 might have the sort of bi-molecular lipoid layer postulated for cell membranes; thicker ones would need some more complex internal arrangement.

The other suggestion about the chemistry of double membranes is that they may contain, or be closely associated with, the cytoplasmic ribonucleic acid (RNA). Dalton suggested this in 1951 on the grounds that their distributions in exocrine pancreas and stomach chief cells roughly coincided with that of cytoplasmic basiphilia; and because basiphilia and double membranes were patchy in normal liver-cells, but more evenly distributed in hepatomas. Bernhard and others (1952), in producing further evidence for this view, have carried out some of the earliest experimental cytology at the electron microscope level. They found that double membranes were more abundant in actively secreting rat livers than in starved livers and in regenerating livers than in non-regenerating livers—exactly like the basiphil component of the ytoplasm. More recently they have found that in centrifuged livers the entrifugal displacement of the double membranes corresponds with that of he basiphilia. Porter (1953) suggests that his endoplasmic reticulum contains RNA, but as his cells were not strongly basiphil, in the way that peptic, xocrine pancreas and regenerating liver-cells are, it is hard to be sure about this. It is, however, noteworthy that strong basiphilia is apparently a feature of his dense particulates, which we believe are more probable counterparts of the double membranes seen in sections.

The evidence that RNA is located in double membranes is at present indirect—and there may always be another cytoplasmic component closely associated with them, confusing the results. Bernhard has pointed out that if the present view is confirmed, it will conflict somewhat with the biochemical bulk centrifugation studies, which indicate that cytoplasmic RNA is located in microsomes—small granules usually said to be 500–2,000 Å in diameter and not markedly anisodiametric. There would be three possibilities: (*a*) microsomes arise from double membranes by disintegration during centrifugation; (*b*) double membranes arise from microsomes during fixation; (*c*) both arise during preparation from some parent structure different from either. The evidence already presented that very similar double membrane pictures are obtained after three different fixatives is in favour of (*a*), but until the RNA content of double membranes is established we cannot be sure that (*a*) is the case.

CONCLUSION

Already in 1853, the first volume of the *Quarterly Journal of Microscopical Science* was concerned with several features of protoplasmic structure discussed here—muscle cytology (Lister) and the nature of flagella (Siebold). Mitochondria, however, had not yet been discovered, and cytologists were only just beginning the great edifice of theories and counter-theories about the structure of 'ground-cytoplasm', which was extensively demolished by Hardy and Fischer. Greatly superior fixation-methods are now available, but still the study of ground-cytoplasm is one of the most thorny corners of cytology. Discussion of it has been avoided here because it is doubtful whether anything useful can yet be said at the electron microscope level of investigation. We question the value of several recent studies in this field, especially of comparisons with relatively simple organic colloids which have very limited relevance to protoplasm. Also regrettable is the emergence of a forest of new terms for describing supposed components of ground cytoplasm—e.g. biosomes, chromidia, and leptons—often poorly defined despite their classical derivation. As Sjöstrand (1953*c*) points out, it is usually easier to preserve (and to check with the light microscope) the structure of the larger and more complex formed elements of protoplasm—mitochondria, cytoplasmic fibrils, and the like. After becoming thoroughly familiar with methods for the faithful preservation of these, we shall be in a better position to turn to the still finer details of the ground cytoplasm. And there is no shortage of formed structures awaiting investigation—the 'bottle-necks' are all technical and two of the foremost are the difficulty of examining serial sections and of applying specific stains. However, in view of the fact that, as recently as 1939, the eminent microscopist, von Ardenne, gave good reasons for believing the whole technique of thin sectioning to be impossible by normal procedures, there are grounds for hoping that these new problems may also be solved in due course.

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The Mechanism of Cell-division

A Study with Carbon Monoxide on the Sea-Urchin Egg

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SUMMARY

1. Developing eggs of the sea-urchin *Psammechinus miliaris* were subjected to arbon monoxide inhibition, which was controlled by changing from green to white ight. The behaviour of the eggs was recorded by time-lapse photography.

2. If inhibition is applied before the eggs enter mitosis, their first cleavage is lelayed by a time which is roughly equal to the period of the inhibition.

3. If the inhibition is applied when the cells have already entered mitosis, they omplete mitosis and cleave with little or no delay, but their *second* cleavage is delayed y a time which is roughly equal to the period of the inhibition.

4. It is suggested that the necessary energy for the second mitosis and cleavage is eing stored up during the first mitosis and cleavage, and that this energy store perates like a reservoir which is continually being filled but siphons out when it is ull. Once the energy has siphoned out, it carries mitosis and cleavage through, even hough the reservoir is not filling up because of carbon monoxide inhibition.

INTRODUCTION

THE effect of respiratory inhibitors and of oxygen lack on cell-division has been investigated by many workers (references in Brachet, 1947). Cells in tissue culture can apparently divide anaerobically; eggs, on the other hand, do not enter mitosis under these conditions, though they can complete a mitosis which is already under way. Much of this work has been done on sea-urchin eggs, and it has been found repeatedly that their division is inhibited by lack of oxygen and by the usual inhibitors of oxidative respiration, namely cyanide, azide, and carbon monoxide (Krahl, 1950). Carbon monoxide is much the most convenient of these inhibitors, for its effect is photo-reversible. Indeed, photo-reversible inhibition with carbon monoxide may be taken to prove that an oxidation process is cytochrome-catalysed. The inhibition is effective in the dark, or in wave-lengths outside the absorption bands of the CO-cytochrome oxidase complex; it is ineffective in white light. Green light is outside the absorption bands, so that by using either green or white light for observation the inhibition can be turned on or off at will. An immediate control of inhibition of this sort is not possible with any other inhibitor; nor, of course, is it possible to switch rapidly from high to low tensions of oxygen.

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In the experiments to be described, batches of fertilized sea-urchin eggs were put in a chamber containing a high partial pressure of carbon monoxide, and the cleavages followed by means of time-lapse photography. The respiratory inhibition was switched on and off at various times, and the behaviour of each egg worked out from the film record. The results give an indication of when, in the mitotic cycle, the primary mechanism for supplying energy is required.

METHODS

The eggs of *Psammechinus miliaris* were used throughout. They were inseminated at sperm densities high enough to ensure that virtually all the eggs were fertilized within 1 minute (Rothschild and Swann, 1951). They were then washed once and transferred to a hanging drop in a moist chamber mounted on a microscope slide. The chamber was then gassed with a mixture of 3 per cent. air (0·6 per cent. O_2, 2·4 per cent. N_2) in CO for 5 minutes. The CO was derived from a pressure cylinder supplied by Messrs. I.C.I., Ltd., and contained less than 0·1 per cent. O_2. The water in the gas bottles in which the mixtures were made up was saturated with ammonium sulphate to ensure that no extra oxygen was taken up. After gassing, the far tap on the moist chamber was closed, but the near tap left open, and a pressure of 6 in. of water maintained throughout the experiment in case of leakage.

The eggs were photographed with a ×3 objective and a ×12·5 eyepiece, by means of a 16-mm. time-lapse camera, taking one frame per minute. The final magnification on the film was about ×10. In this way, fields of about 50–80 eggs could be photographed comfortably. A Pointolite was used as a light source with a piece of ON 20 glass to absorb the heat. For photography during inhibition, a Farrand Interference filter (525 mμ) and a 1/80th neutral filter were put in front of the lamp. For photography without inhibition, these were removed and a 1/1600th neutral filter put between the microscope and the camera. This latter arrangement gave a rather denser negative, so that the switching on and off of the inhibition was automatically recorded.

The films were analysed with a cine-viewer, and the frame number at which each egg began its first, and in some cases second cleavage was recorded. The criterion used throughout for the start of the cleavage was the appearance of a distinctive furrow or V. This criterion, unlike elongation, has the merit that it can be spotted even when the axis of the cleaving egg is in line with the axis of vision.

A word of explanation is required about the particular gas mixture used—0·6 per cent. O_2, 97 per cent. CO. It was known from the work of Clowes and Krahl (1940) that O_2 tensions of about 0·7 per cent. were needed to get complete inhibition in darkness or green light, while O_2 tensions of 0·4 per cent. gave inhibition under all conditions. It was clearly necessary, therefore, to work somewhere in this range, and after some trials 0·6 per cent. O_2 was chosen. It gave complete inhibition in green light, while in white light it allowed eggs to develop normally and in step with the controls for many hours.

In addition to experiments with batches of eggs, a few observations were ade on single eggs by polarized light, to determine the particular stage in e mitotic cycle at which development was arrested under inhibition.

RESULTS

The results of five series of experiments, each involving eggs from a ifferent female, are summarized in table 1. The times at which eggs started

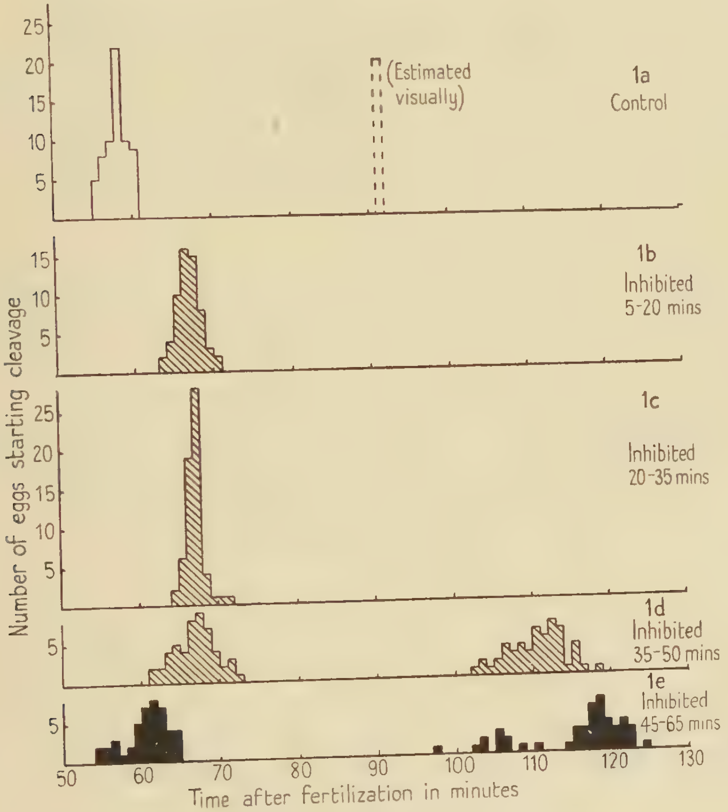

FIG. 1. Results of experiments—Series 1.

to cleave are shown diagrammatically in figs. 1–5. It will be seen from an examination of the times of first cleavage that the eggs fall into two groups, depending on when the inhibition is applied; either they are delayed by an amount roughly proportional to the length of time over which the inhibition is operative, or they are delayed little if at all. In the diagrams, eggs which are delayed little if at all are shown in black, while eggs which are delayed substantially are shown hatched. Where the experiment was continued long enough to determine the time of second cleavage, the eggs which were shown in black originally are shown in black again; those which were

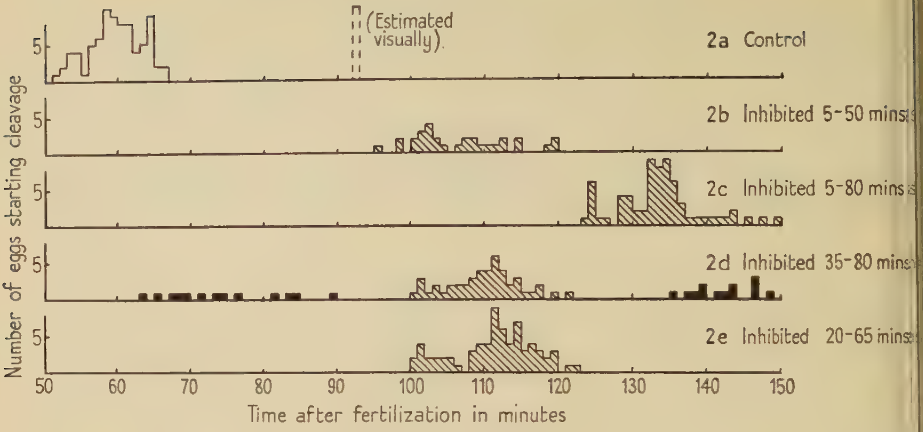

FIG. 2. Results of experiments—Series 2.

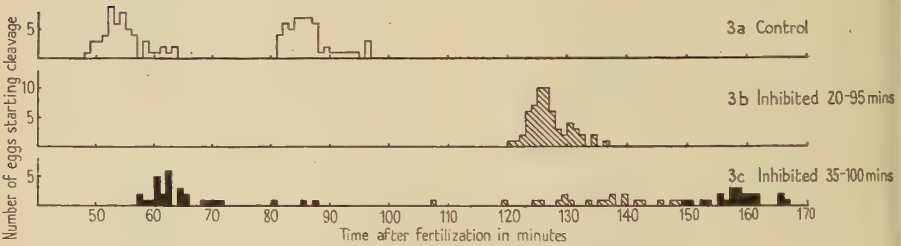

FIG. 3. Results of experiments—Series 3.

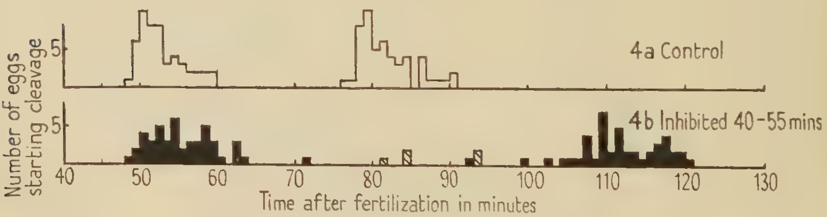

FIG. 4. Results of experiments—Series 4.

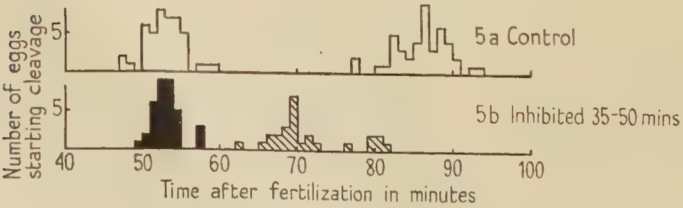

FIG. 5. Results of experiments—Series 5.

TABLE I

Series	Time of Inhibition	Duration of inhibition	Time of first cleavage	Delay of first cleavage	Time of second cleavage	Time from first to second cleavage	Delay of second cleavage	Number of eggs counted	Percentage of eggs cleaving	Temperature ° C.
1 *a*	Control	Nil	57·8	..	(91)	33·2	..	64	100	18·75
b	5–20	15	66·4	8·6	..	..	..	60	100	19·0
c	20–35	15	66·6	8·8	..	..	..	62	100	19·0
d	35–50	15	66·4	8·6	110·0	43·6	10·4	51	94	18·8
e	45–65	20	60·1	2·3	115·9	55·8	22·6	46	100	19·0
2 *a*	Control	Nil	59·2	..	(92)	32·8	..	80	97·5	19·0
b	5–50	45	106·2	47·0	..	..	..	30	96·7	18·3
c	5–80	75	133·0	73·8	..	..	..	67	95·5	18·4
d	35–80	45	74·1 109·9	14·9 50·7	141·8 ..	67·7 ..	34·9 ..	61	98·4	19·0
e	20–65	45	110·9	51·7	..	..	..	69	100	19·0
3 *a*	Control	Nil	54·1	..	85·9	31·8	..	53	98·1	20·2
b	20–95	75	126·4	72·3	..	..	..	60	98·3	19·8
c	35–100	65	64·7 133·3	10·6 79·2	157·8 ..	93·1 ..	61·3 ..	49	100	19·4
4 *a*	Control	Nil	52·3	..	81·3	29·0	..	51	94·1	20·7
b	40–55	15	55·2 [87·0]	2·9 [34·7]	111·5 ..	56·3 ..	27·3 ..	51	100	20·4
5 *a*	Control	Nil	52·5	..	85·6	33·1	..	44	99·9	20·2
b	35–50	15	52·6 70·7	0·1 18·2	96·0 107·6	43·4 36·9	10·3 3·8	61	100	20·2

NOTE: Figures in round brackets are based on visual estimates. Figures in square brackets are based on five eggs only.

shown hatched are shown hatched again. It should be noted that eggs whic cleaved once did not always cleave a second time before the end of the exper ment, so that the numbers of eggs in corresponding groups do not alway tally precisely. This point is discussed later. It should also be noted that th figures in table 1, and the polygons in figs. 1–5, refer to the times at whic eggs *started* to cleave. In some cases they failed to complete cleavage an slowly regressed. This point is also discussed later.

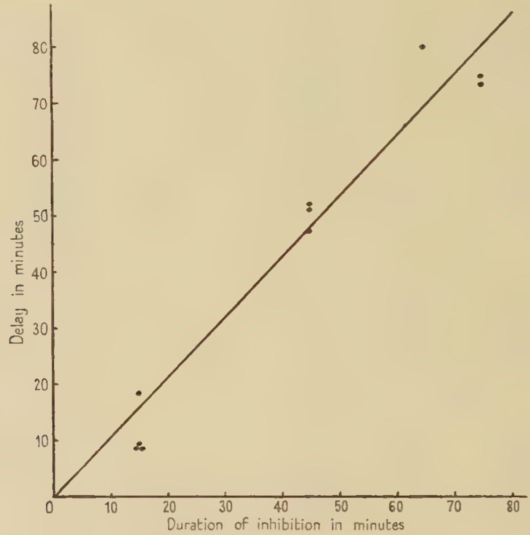

Fig. 6. The delay of first cleavage and the duration of inhibition.

The temperature varied a little in the different experiments of each series and rather more seriously between different series. It would be possible t correct for this to some extent by using the known Q_{10} of sea-urchin eg cleavage. But the validity of this procedure is rather doubtful in such experi ments as these, and no attempt has in fact been made to apply corrections In any case, batches of eggs from different females vary somewhat in thei time of cleavage, even when kept at the same temperature.

It will be seen from the control experiments (1*a*, 2*a*, 3*a*, 4*a*, and 5*a*) tha the mean time of first cleavage is between 52 and 59 minutes after fertilization When inhibition is applied in the early stages of development (between 5 an 20 minutes after fertilization) cleavage does not take place at the norma time. Indeed, if the inhibition were kept on permanently the eggs woul never cleave. When, however, the inhibition is lifted, the eggs do cleave i due course. This can be seen from experiments 1*b*, 1*c*, 2*c*, and 3*b*. The cleave, moreover, with a delay over normal cleavage that is roughly equal t the length of the inhibition period. Length of inhibition and length of induce delay are plotted in fig. 6.

If the inhibition is applied in the later stages of mitosis, however, as is wel own, cleavage goes through more or less normally. This is evident in 1*e*, iere inhibition began at 45 minutes and the delay over normal cleavage is ly slight. Where the inhibition begins earlier than about 45 minutes, the uation becomes more complicated. It might be expected that progressive vancing of the start of inhibition would gradually increase the delay. But, fact, the two types of behaviour—little or no delay, and delay comparable the period of inhibition—appear simultaneously. As the start of inhibition moved forwards, the proportion of eggs showing the first type of behaviour creases, and the proportion showing the second type increases.

In experiment 4*b*, for instance, with inhibition starting at 40 minutes, all it 4 eggs cleaved with only slight delay. Eggs inhibited from 35 minutes show nsiderable variability. In experiments 3*c* and 5*b* the majority show little elay. In 2*d*, on the other hand, the majority show substantial delay. In 1*d*, o eggs at all cleave at the normal time. This variability is to be expected, owever, from the temperature during the different experiments, which was ighest in 3*c* and 5*b* and lowest in 1*d*. The time at which the inhibition starts 35 minutes in this case) corresponds, of course, to a later stage of development, the higher the temperature.

In all the experiments where inhibition begins late in the mitotic cycle, one urious effect is apparent, namely, that the process of cleavage itself is appreiably slowed up. In extreme cases it never gets more than about a quarter r half-way through, and is followed by a slow regression of the furrow. his effect is apparent from the figures of table 2, which give the average ime (based on 20 suitably oriented eggs) between the first signs of elongation, nd the appearance of a distinct furrow or V (the criterion used for the start f cleavage in the results so far presented).

TABLE 2

Experiment no.	*Average time from elongation to beginning of furrow*
1*a* (control)	1·5
1*e*	2·6
2*a* (control)	1·5
2*d*	5·1 (8 eggs only)
3*a* (control)	1·4
3*c*	3·7
4*a* (control)	1·4
4*b*	2·4
5*a* (control)	1·5
5*b*	2·1

It is noticeable that this slowing-up of cleavage is most marked when th inhibition starts relatively early, and continues over the period of cleavag (2*d* and 3*c*). It is least marked when the inhibition starts late or is lifte before cleavage actually takes place (1*e*, 4*b*, 5*b*). It is evident that if it wer possible to measure the very first onset of cleavage, as opposed to the appearance of the furrow, the recorded delays would be even less than they are.

In a number of experiments the effect of inhibition on the time of th second cleavage was investigated. This was only done twice for eggs whic

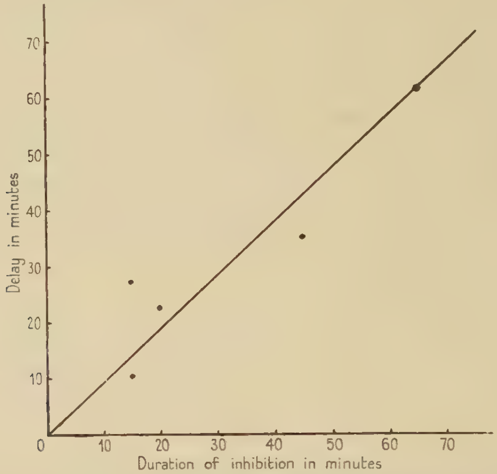

FIG. 7. The delay of second cleavage and the duration of inhibition. (The delay is taken as the time from first to second cleavage, less the time from first to second cleavage in the controls.)

had suffered a substantial delay in first cleavage (1*d* and 5*b*). In these cases delays in second cleavage of about 10·4 and 3·8 minutes were produced. The delay in second cleavage for eggs that had been inhibited sufficiently late to suffer little or no delay in first cleavage is more striking, and *corresponds closely once again to the duration of inhibition*, although the inhibition took place, at least partially, during the first mitotic cycle (1*e*, 2*d*, 3*c*, 4*b*, and 5*b*). These results are plotted in fig. 7. Delay in second cleavage is taken as being the time from first to second cleavage less the time from first to second cleavage in the controls.

The pattern of cleavage after inhibition is not always normal and regular. When the inhibition begins early cleavage is relatively tidy, but when it begins at 35 or 40 minutes the delayed cleavages are invariably multiple and very irregular. The undelayed cleavages, on the other hand, are regular, though, as was pointed out earlier, they frequently regress. The subsequent cleavage of these regressed eggs is nevertheless normal, though they quite often divide into two and not into four. It is to be presumed that in the delayed eggs the centrosome mechanism is substantially deranged.

One last point calls for comment, namely, that when inhibition is relatively te, so that some eggs cleave or attempt to cleave without much delay, there a marked difference in the time of second cleavage as between those eggs hat succeeded in cleaving the first time and those that regressed. The ggs that succeeded in cleaving divide the second time markedly *later* than hose that regressed. This was apparent in experiment 3*c*, when 29 eggs leaved or attempted to cleave. Twenty-three of the 29 regressed, and 22 of hem had cleaved by the end of the record at 169 minutes, their average time f second cleavage being 133·3 minutes. On the other hand, 6 of the 29 succeeded in cleaving the first time, and none of them had cleaved a second ime by 169 minutes.

The results of observing eggs in polarized light are straightforward. When n egg is inhibited during interphase, that is, when it shows only a sperm ster, it progresses no farther. The nucleus does not swell, the characteristic streak stage never appears, and the spindle and asters do not develop. On the other hand, the sperm aster does not regress. The egg as a whole tends to become rather wrinkled. When the inhibition is switched off the egg rapidly rounds up, and the normal stages of mitosis follow on. When the egg is inhibited at a later stage—that is to say, during metaphase, anaphase, or telophase—mitosis continues, though the mitotic figure may be rather small, and cleavage may fail to complete. In due course interphase asters develop, and persist as long as the egg is inhibited, without fresh mitotic figures appearing. When the egg is inhibited between the sperm aster stage (interphase) and metaphase—that is to say, when it is showing a streak or very small asters and spindle—it may follow one of two courses: either it develops no farther, and even regresses slightly to give a typical-looking sperm aster; or it continues but forms only small asters and spindle and cleaves rather slowly, often failing to cleave completely. Where mitosis continues, the egg finally ceases development in the next interphase.

Discussion

It is evident that inhibition of the cytochrome oxidase system, and hence shutting off the supply of energy derived from aerobic metabolism, if applied before an egg has entered mitosis, prevents it from entering mitosis. There are thus two possibilities: that the egg continues developing up to the end of interphase in the absence of the normal energy supply, but then comes to a stop; or that it stops developing at once. The results of the present experiments suggest strongly that it follows the second of these courses, for if it followed the first course there would be a fixed time between the end of inhibition and the onset of cleavage, and it is evident that this is not the case. On the contrary, there is a close relation between the duration of inhibition and the delay in cleavage that ensues.

If, on the other hand, an egg has already entered mitosis when inhibition begins, it will continue developing. If it has reached metaphase or anaphase it will cleave with little or no delay. If it has only reached prophase it will cleave or attempt to cleave, but the actual process of cleavage will be slow, which will result in a certain delay. In either case, however, the *second* cleavage will be delayed by an amount roughly equal to the total period of inhibition. It follows that the necessary preparations for the second cleavage are going ahead during the first cleavage.

The present experiments throw no light on what the necessary preparations are, but there would seem to be two main possibilities: either the cell has to store up energy derived from aerobic metabolism, and this store, when it is full, starts off the next mitosis, regardless of whether aerobic metabolism is going on or not; or the cell has to synthesize and store up some mitotic controlling substance, not itself a carrier of energy, which starts off mitosis when it reaches a certain level, the energy for this synthesis being supplied by aerobic metabolism, while the energy for mitosis is supplied by anaerobic metabolism. It is not possible to distinguish between these two schemes at present, but on grounds of simplicity the first possibility seems the more attractive.

Assuming, then, that an energy store exists, it must behave rather like a reservoir with a siphon. The reservoir starts to fill after fertilization, and is filled in about 35 minutes. The contents then siphon out, and precipitate mitosis and cleavage, which take a further 20 minutes, being complete by 55 minutes. The emptied reservoir, however, continues to fill while mitosis is in progress. The reservoir is presumably not full by the end of cleavage; in fact it might reasonably not be expected to be full until about 35 minutes had elapsed from its first emptying, that is, until about 70 minutes. It is significant that this is just about the time that the second mitosis begins. This then takes some 20 minutes to complete, and the second cleavage occurs at about 90 minutes. On such a scheme it is to be expected that successive mitoses and cleavages would be separated by the period of time required for the reservoir to fill, i.e. 35 minutes, and this is actually the case (Gray, 1931). Cleavages in the sea-urchin egg are spaced rather regularly at about 35-minute intervals for a considerable time. The first cleavage, on the other hand, will necessarily take longer, for the reservoir has to fill, and the first mitosis has then to run through.

Though some such scheme as this accounts satisfactorily for the main findings of the present paper, it does not account for the rather curious point that when cleavage occurs during inhibition (i.e. when the inhibition has been applied after the start of mitosis), the actual process of cleavage is appreciably slowed. It might be supposed that the reservoir would not initiate mitosis until it contained enough energy to see the process through, but this does not seem to be the case. Mitosis can apparently start off without enough energy to carry it through. The most obvious explanation would seem to be that the primary supply of energy is not wholly taken up by the reservoir, but that secondary mechanisms utilizing the reservoir energy for the mitotic process also receive directly some of the primary supply. When this is cut off, the completion of mitosis and cleavage becomes precarious. However, it would be possible to account for the facts in a variety of other ways—by the accumulation of toxic products, for instance.

The scheme offers no explanation either of the curious finding that in eggs hich cleave or attempt to cleave during inhibition, the second cleavage is ore delayed in those eggs that succeed in their first cleavage than in those at fail and subsequently regress. The most likely explanation would seem be that an egg which succeeds in cleaving exhausts its available energy pplies more completely than one which fails. This is not improbable from physical point of view. But it would imply that such energy as was saved y the egg failing to cleave was transferred back to the reservoir. It is idle speculate further on the point at present.

The main conclusion that emerges from these experiments is that the cyto-hrome system is normally active, and therefore providing energy for mitosis nd cleavage, *throughout early development*—both during interphase and uring mitosis. This is quite consistent with results from manometric studies n respiration, which have shown repeatedly that aerobic respiration is more r less constant throughout the early cleavage stages. The fluctuations found ecently by Zeuthen (1950) and Scholander and others (1952) are too small o invalidate such a conclusion.

A final word is necessary on the subject of the energy reservoir. The natural uggestion for such a reservoir is, of course, ATP; but the amount of ATP n the sea-urchin egg is not great, and it does not appear to fluctuate (Cleland, 953). Moreover, the energy in the postulated reservoir is not, as ATP pparently is, free and readily available; it is very specifically locked up until uch time as it siphons out and triggers off mitosis. It may be, therefore, hat we have got to look elsewhere for the energy reservoir, and to look articularly for a store which is not available to the cell until it becomes full p. The fact that this energy store is not normally available for general use s particularly apparent from the fact that delay times are so nearly equal to nhibition times. If it were generally available it would be depleted during nhibition, and the delay in cleavage would always be greater than the period f inhibition; but this is not so, at least up to 75 minutes.

I should like to thank the Director and staff of the Marine Station, Mill-ort, Scotland, for their customary kindness and help, and Lord Rothschild or drawing my attention to the possibilities of the technique employed and or lending me apparatus.

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Measurements on Sea-urchin Eggs with an Interference Microscope

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With one plate in black and white (fig. 1) and one plate in colour (fig. 2)

SUMMARY

Observations and measurements with an interference microscope on sea-urchin eggs have established the following points.

(1) The refractive index (R.I.) of the cytoplasm of the unfertilized egg is 1·375, which is equivalent to a concentration of solid matter of about 25 per cent. Very similar values were obtained from eggs 10 minutes after fertilization.

(2) There may be a small drop in the R.I. during the interphase before the first cleavage and during mitosis, but this is not greater than about 0·2 per cent., which is equivalent to a change in concentration of 2 per cent.

(3) The nucleus of the unfertilized egg has an R.I. of 1·360, equivalent to a concentration of 16 per cent.

(4) The R.I. of the mitotic figures is lower than that of the cytoplasm, by a ratio of 0·89 in metaphase. This is probably due to the absence of granules in the mitotic figures, since a similar ratio is obtained between the clear and the granular regions in centrifuged eggs.

(5) In metaphase, the chromosomes have the same R.I. as the spindle and are invisible. During the later stages of mitosis their R.I. drops, and they become visible.

(6) The dry thickness of the fertilization membrane is about 160 Å. There is evidence of a colloid in the perivitelline space at a concentration of about 0·07 per cent.

(7) The jelly has a concentration of less than 0·0055 per cent.

INTRODUCTION

THE interference microscope resembles the phase contrast microscope in rendering visible changes in the refractive index (and thickness) of objects. In physical terms, it converts phase differences to amplitude differences. It is a rather more complicated and expensive instrument than the phase-contrast microscope, but it has many advantages. The contrast between phase objects can be varied at will and, with white light, can be shown as striking colour changes which resemble those in a stained specimen. It is possible to detect small continuous changes in refractive index (R.I.), whereas phase-contrast will, in general, only reveal sharp discontinuities. Some forms of interference microscope are more sensitive than phase-contrast, and also lack the objectionable halo round the edge of phase-contrast images. Above all, the interference microscope has the great advantage that it can be used for quantitative work.

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It measures the product of R.I. and thickness, and, if the thickness is known, the resulting R.I. gives the concentration or dry weight of matter in the object (Davies and Wilkins, 1952). This will be only an approximate figure with most living material where the composition and homogeneity are doubtful, but it could be extremely accurate with a material where the relation between concentration and R.I. was known. An interference microscope can measure all to at least a hundredth of a wavelength retardation, which, in an object 100 μ thick, represents a difference of absolute protein concentration of about 0·03 per cent.

METHODS

Since the interference microscope is still a relatively novel instrument for biologists, it is perhaps worth giving a very brief description of the instrument and the formulae that are used with it. The microscope we used was a prototype of a model which is being manufactured by Messrs. C. Baker Ltd. and which will shortly be available. Various patterns of laboratory-made interference microscopes have been used in the last few years, but, as far as we know, this is the first one to be available commercially. The instrument is basically a polarizing microscope with the addition of quartz and calcite plates on the front of the condenser and objective. The effect of the condenser plates is to split the incident polarized light into two beams with their planes of polarization at right angles. One beam passes through the object and the other beam (the reference beam) passes through the background or surrounding medium. The two beams are recombined in the objective and, if the object beam has suffered a phase retardation (due to the R.I. of the object being higher than that of the medium), the resultant beam will be elliptically polarized. The amount of retardation is measured by a compensator and analyser, as in an ordinary polarizing microscope (in this instrument, by a quarter-wave plate and a rotating analyser).

In the prototype we used, there were two different systems for splitting the incident light. The 16-mm. objective worked on a 'sheared beam' system in which the reference beam went through the object plane parallel to the object beam but separated from it by a horizontal distance of about 330 μ. The 4-mm. objective worked on a 'double focus' system where the reference beam was focused above the object plane, and was therefore spread out into a broad out-of-focus pattern in the object plane. The 'double focus' system was easier to set up initially, but in every other way was less satisfactory. It was less sensitive than the 'sheared beam' system and also produced an objectionable halo round the object, similar to that produced by phase-contrast, though less noticeable. The 'sheared beam' system had the further great advantage that the initial retardation between the two beams could be altered over a range of about ten wavelengths by tilting the condenser. This meant that the object-medium retardation could be set at any desired point on the spectral order (e.g. by setting the initial retardation at the 1st order purple in white light, the nucleus of a sea-urchin egg would appear red and the cytolasm blue); and also that it was possible to measure large retardations beyond he limits of the compensator ($\pm\lambda/2$). We are told, however, by Messrs. Baker hat they are going to improve the 'double focus' system.

If Γ is the retardation of an object, and t its thickness, then the difference etween the R.I. of the object (n_o) and the R.I. of the medium through which he reference beam is passing (n_m) is given by:

$$n_o - n_m = \Gamma/t$$

This, of course, gives n_o if n_m is known.

f C_o is the percentage concentration of material in the object (dry weight in vater), and n_w is the R.I. of water, then:

$$C_o = 100\,(n_o - n_w)/\chi,$$

vhere χ is 100 α, and α is the specific refractive increment of the material in solution. Davies and Wilkins (1952) give values of χ for a number of cell constituents, and emphasize the important point that it does not vary much between the major cell constituents (proteins, fats, and carbohydrates). It seems reasonable, therefore, for the purpose of this paper to take the limits of χ as 0·14 and 0·19, with an average value of 0·165. As Davies and Wilkins point out, χ varies a little with C_o, but, since the value of χ is only approximate, this variation can probably be ignored.

The observations and measurements were done on the eggs of *Psammechinus miliaris*. When necessary, the fertilization membranes were removed by passing the eggs through fine mesh bolting silk just after fertilization.

If uncompressed sea-urchin eggs under a supported coverslip were observed with the 16mm. 'sheared beam' objective, they showed a series of conspicuous interference rings due to the increase in thickness on moving towards the centre (fig. 1, A). These rings obscured the internal structures, so for the purpose of observation it was better to work with compressed eggs. The eggs were compressed by placing them under a coverslip which rested on the slide at one side, and was supported by a piece of No. 0 coverslip at the other side. The best region for observation was where the eggs were compressed to a diameter of about 200 μ (twice normal) and a thickness of about 25 μ. This degree of compression did not stop development, yet it gave an area of constant thickness over most of the egg in which differences of retardation were due to differences of R.I., rather than to differences in thickness, as in the uncompressed eggs. This arrangement was unsuitable for observing slow changes in R.I. over a period of minutes. Evaporation of water from under the coverslip bent it down onto the slide and decreased the thickness of the compressed eggs. This made it difficult to say whether a change in retardation was due to a change in thickness or to a change in R.I.

OBSERVATIONS

When compressed, the most noticeable feature of the mature unfertilized egg was the nucleus. By altering the compensator or tilting the condenser when using white light, the nucleus could be made to show a striking colour-contrast against the cytoplasm, e.g. a red nucleus against a blue cytoplasm (fig. 2, B), or conversely. For measurement or photography it was best to use monochromatic light (green light of 5500 Å wavelength). In this case the nucleus could be made either dark against a light background (fig. 1, B) or light against a dark background (fig. 1, C). The sequence of colours in white light showed that the R.I. of the nucleus was less than that of the cytoplasm. The cytoplasm showed a roughly uniform retardation over most of the egg, though there were small variations due to the granules. At the edge there was a complex pattern of rings mainly due to the sharp decrease of thickness, and therefore retardation, where the surface of the egg curved away from the cover-slip. Without a very detailed analysis of this pattern, it would be impossible to say whether the cortex had a different R.I. from the cytoplasm. The jelly round the outside of the egg produced no detectable retardation.

In the case of the oocyte (figs. 1, D and 2, A) the germinal vesicle had a lower R.I. than the cytoplasm, while the nucleolus had a higher R.I. than the cytoplasm. In some unfertilized eggs there were one or more patches of low R.I. either round the nucleus (figs. 1, E and 2, B) or elsewhere in the cytoplasm. It seems reasonable to suggest that such eggs had only just changed from oocytes and that these patches were the remains of the dilute contents of the germinal vesicle. It is interesting that these patches, presumably of low concentration, remained more or less constant in size for many minutes. In a fluid system of such a small size, diffusion should even out concentration differences very rapidly; so it seems likely that the inside of the egg is not as fluid as viscosity measurements would suggest.

On fertilization there were no obvious changes in the cytoplasm or nucleus until the 'streak' stage, when the 'streak' itself appeared as a structure of low R.I. surrounding the nucleus (fig. 1, F). The asters and spindle which were evident in metaphase appeared as a sharply defined 'dumb-bell' of low R.I. (figs. 1, G and 2, C), similar to the faint figure which can be seen on defocusing an ordinary microscope with a stopped-down iris (this is because an ordinary microscope, when defocused, will reveal phase changes). As the asters spread through the egg during anaphase (fig. 1, H) the sharp difference between the R.I. of the cytoplasm and that of the mitotic figure was evened out. By the beginning of cleavage, the reformed daughter nuclei appeared as regions of even lower R.I. than the mitotic figure. Fig. 1, J, which shows this, may appear slightly misleading, since both the nuclei and the cytoplasm are lighter than the asters. This is because the asters were compensated to blackness to render them visible, so the nuclei, which have a lower retardation still, were in a different Newtonian order from the cytoplasm. Fig. 1, J and K show dark patches and streaks in the furrow. These were due both to the decreasing

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FIG. 2 (plate). 'Kodachrome' photographs with 16mm. interference objective. A–E are compressed eggs of *Psammechinus miliaris*. In all except A, high R.I. material is blue, and low R.I. material is red or yellow. A. Oocyte. B. Unfertilized. C. Metaphase. D. Cleavage. E. Cleavage. F. 'Spit cells'.

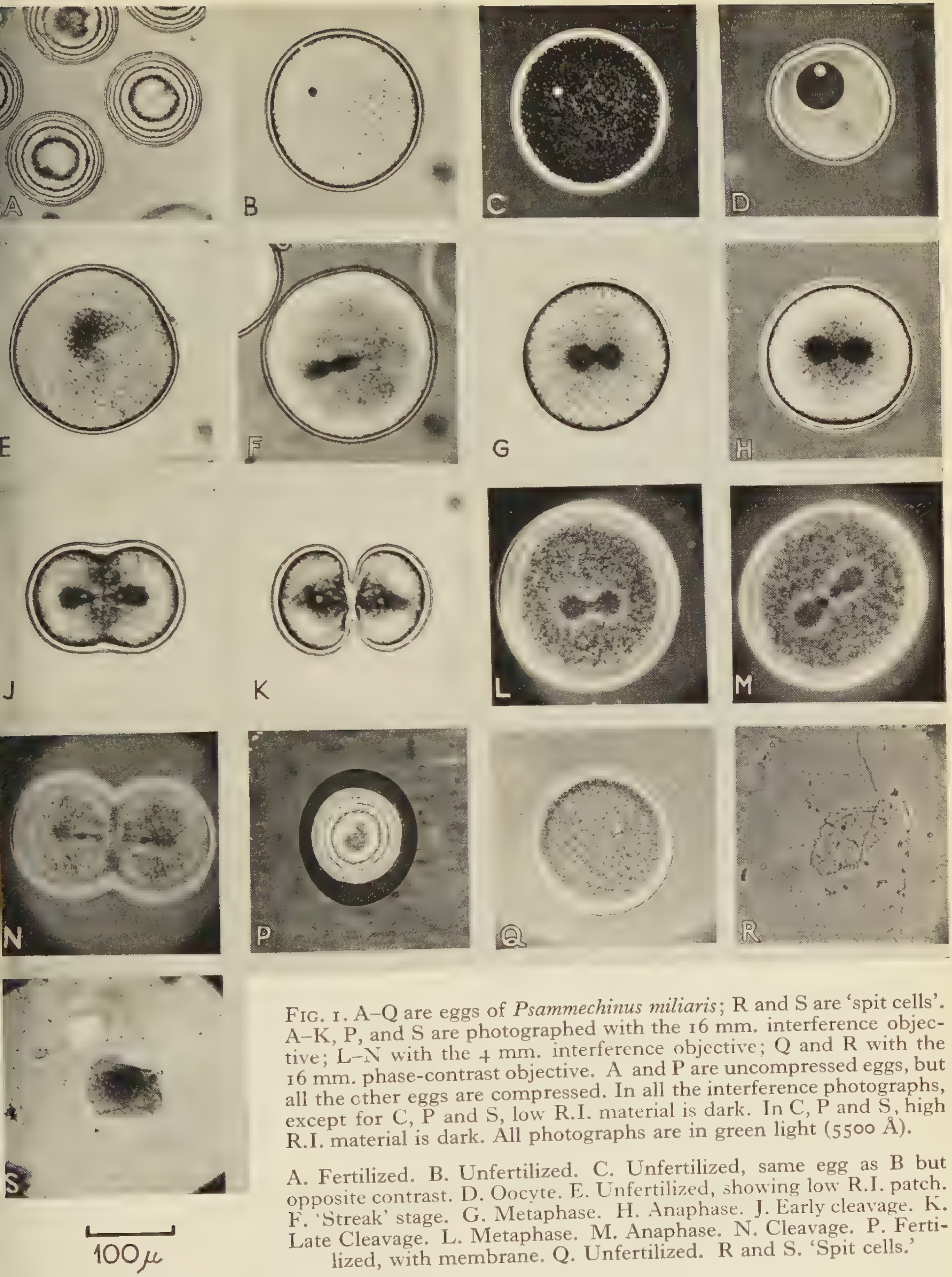

FIG. 1. A–Q are eggs of *Psammechinus miliaris*; R and S are 'spit cells'. A–K, P, and S are photographed with the 16 mm. interference objective; L–N with the 4 mm. interference objective; Q and R with the 16 mm. phase-contrast objective. A and P are uncompressed eggs, but all the other eggs are compressed. In all the interference photographs, except for C, P and S, low R.I. material is dark. In C, P and S, high R.I. material is dark. All photographs are in green light (5500 Å).

A. Fertilized. B. Unfertilized. C. Unfertilized, same egg as B but opposite contrast. D. Oocyte. E. Unfertilized, showing low R.I. patch. F. 'Streak' stage. G. Metaphase. H. Anaphase. J. Early cleavage. K. Late Cleavage. L. Metaphase. M. Anaphase. N. Cleavage. P. Fertilized, with membrane. Q. Unfertilized. R and S. 'Spit cells.'

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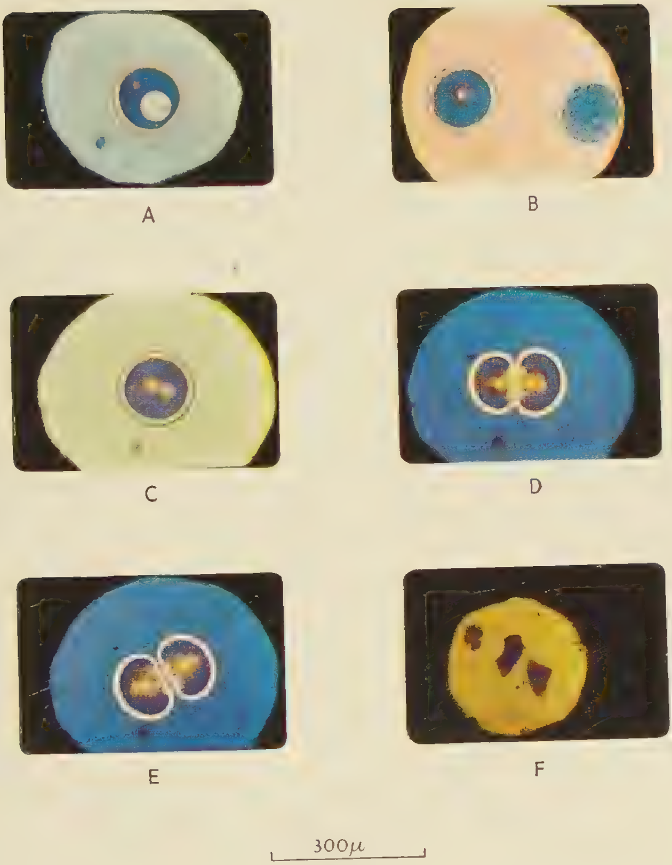

FIG. 2. 'Kodachrome' photographs with 16mm. interference objective.

A–E are compressed eggs of *Psammechinus miliaris*. In all except A, high R.I. material is blue, and low R.I. material is red or yellow. A. Oocyte. B. Unfertilized. C. Metaphase. D. Cleavage. E. Cleavage. F. 'Spit cells'

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ickness in this region and also to the wrinkles which normally occur there. ig. 2, D and E show cleaving eggs in colour.

For observation of the finer details of mitosis it was necessary to use the igher resolution of the 4-mm. objective, even though the 'double focus' ystem gave a much lower contrast. This showed that the chromosome groups ere invisible in metaphase (fig. 1, L) and must therefore have been of the me R.I. as the spindle. They became visible in early anaphase as clumps of lower R.I. than the spindle (fig. 1, M), and then concentrated in late anaphase form the daughter nuclei which were smaller in size but lower in R.I. than he original clumps (fig. 1, N). It can also be seen from these photographs that he astral rays are of lower R.I. (i.e. darker) than the cytoplasm.

The fertilization membranes were removed from the fertilized eggs since hey produced wrinkles and irregularities in the eggs when they were com- ressed. Observations on uncompressed eggs with intact membranes showed hat there was a definite retardation on looking through the membrane and erivitelline space (fig. 1, P). Measurements of this are described later.

We think it worth while including three photographs which compare inter- erence and phase-contrast. Fig. 1, Q was taken with a 16-mm. phase-contrast bjective (Cooke, Troughton, and Simms) and should be compared with ig. 1, C. The remaining two photos are of 'spit cells' (epithelial lining cells f the mouth), fig. 1, R being taken with the 16-mm. phase objective, and fig. 1, S being taken with the 16-mm. interference objective. The higher contrast with the interference system is very obvious. Fig. 2, F shows 'spit cells' in colour under the 16-mm. interference objective.

MEASUREMENTS

The R.I. of the cytoplasm of an unfertilized egg was found by measuring the retardation through the middle of the uncompressed egg (using the condenser tilting adjustment to get to the nearest wavelength, and then measuring fractions of a wavelength with the compensator). This retardation was about 7 to 8 wavelengths. Round eggs were chosen for measurement, and the diameter in the vertical plane was assumed to be the same as that measured in the horizontal plane. The R.I. and the concentration of material within the egg were then worked out from the equations given earlier. The limits of χ were taken as 0·14 and 0·19, with an average value of 0·165. An average from five eggs was taken for this and subsequent measurements. The results are shown in table 1, together with a similar measurement for eggs 10 minutes after fertilization. It can be seen that there is no significant difference between them.

Table 1 also includes a figure for the nucleus of the unfertilized egg. This was found from compressed eggs by measuring the retardation of the nucleus against that of the cytoplasm to get the R.I. difference between them, and then subtracting this difference from the difference between cytoplasm and sea-water in the first row of table 1.

TABLE I

	(1) *Diff. of R.I. from sea-water*	(2) σ *of* (1)	(3) *Absolute R.I.* (1)+1·339	(4) *Concentration per cent. by wt. of dry matter in dist. water*		
				Limits		*Average*
				$\chi = 0{\cdot}14$	$\chi = 0{\cdot}19$	$\chi = 0{\cdot}165$
Cytoplasm (unfert.) .	0·036	0·002	1·375	30·0	22·1	25·5
Cytoplasm (fert.) .	0·037	0·001	1·376	30·7	22·6	26·1
Nucleus (unfert.) .	0·021	0·003	1·360	19·3	14·2	16·4

In order to follow the changes in the R.I. of the cytoplasm of developing eggs it was necessary to use a method which avoided measuring the R.I. of the mitotic figures, since the observations had shown that they had a lower R.I. than the outer cytoplasm. The method described above for the unfertilized egg was unsuitable, since it measured the retardation through the centre of the egg, and therefore through the middle of the mitotic figure. Another method was used which gave the relative changes in R.I. for the outer regions of the cytoplasm. This involved photographing developing eggs every 4 minutes with a constant setting of the compensator, and then measuring from the photographs of each egg the diameter of one of the interference rings (x) and the diameter of the egg (d). It can be shown by simple geometry that the R.I. of the region of the egg under the ring is proportional to

$$1/\sqrt{(d^2-x^2)}.$$

The ring chosen for measurement was the second from the centre in the eggs shown in fig. 2, A. This ring was always about two-thirds of the egg diameter; the R.I. was thus being measured for a region of the cytoplasm about two-thirds of the way from the centre to the outer edge of the egg, which avoided the mitotic figure except in the final stages of anaphase when the asters reach to the outside of the egg. The measurements had to be stopped at this stage in any case since the shape of the egg was changing. The results for three eggs are shown in fig. 3. Although the measurements give only the relative changes in R.I., the absolute R.I. has been put in by assuming that the eggs start with an average R.I. of 1·376 (from table 1). There appears to be a steady fall in the R.I., but only a small one—an average of about 0·2 per cent. It is possible that this represents a real change in R.I., but it may be that the fall is in fact due to an increase in the measured diameter of the egg (rather than to an increase in the ring diameter) which might have been caused by slight changes in the microscope focus. It seems reasonable to conclude that there may be a fall in R.I. during interphase, but that it is not greater than 0·2 per cent. (equivalent to a change in concentration of about 2 per cent.).

Measurements on metaphase and early anaphase asters showed that the ratio of the R.I. differences of the low R.I. interior of the asters and the higher

.I. cytoplasm [i.e. $(n_{\text{aster}}-n_m)/(n_{\text{cyt}}-n_m)$] was 0·89. It is likely that this ifference is due mainly to the expulsion of high R.I. granules from the centre f the asters. This suggestion was tested by measurements on centrifuged nfertilized eggs (5,000 g. for 20 minutes in a sugar gradient). The strip of lear cytoplasm at the centripetal end had a lower R.I. than the granular entrifugal end, and the ratio of the R.I. differences was 0·83. This is much he same as the ratio for the asters to the cytoplasm.

Isolated fertilization membranes were prepared by crushing eggs under coverslip about 10 minutes after fertilization. The retardation of a double thickness of membrane was about 4° (61 Å with green light), but, since this is near the limit of measurement of the compensator there was a possible error of perhaps ±2°. Assuming that this membrane is composed of protein with an R.I. of 1·525 (that of gelatin), the *dry* thickness of the single membrane would be 160 Å±80 Å.

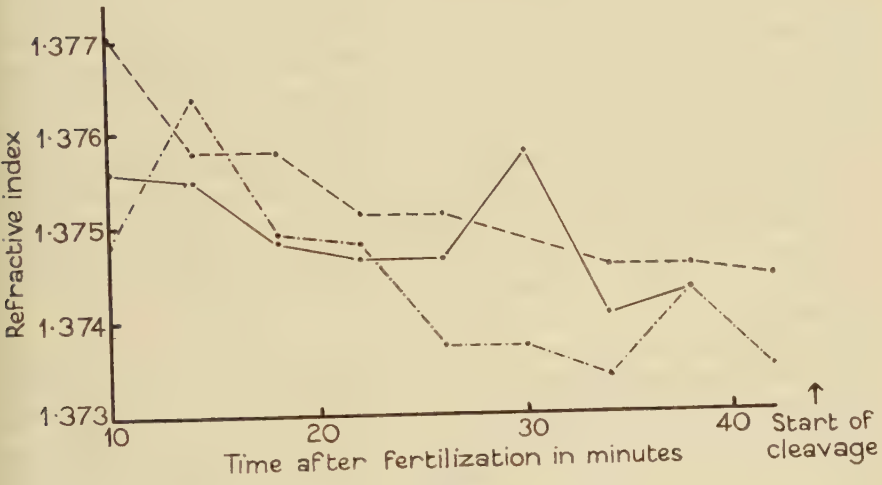

FIG. 3

Measurements were also made through the fertilization membrane and the perivitelline space in intact eggs. For this purpose it was better to use eggs which had been left to stand for several hours and which, when fertilized, lay in an eccentric position within their fertilization membranes and did not obstruct the vertical line through the centre of the membrane. In such cases the retardation through the centre of the egg was due to the combination membrane-perivitelline-space membrane, and the thickness could be taken as the same as the horizontal diameter of the membrane. The average retardation on ten eggs was 244 Å ($\sigma = 64$ Å). Subtracting 61 Å for the double thickness of membrane, this gave the retardation of the fluid in the perivitelline space as 183 Å over and above sea-water. Over 150 μ (the average membrane diameter) this is equivalent to an R.I. difference from sea-water of $1{\cdot}22 \times 10^{-4}$, and a concentration of material, presumably colloidal, in excess of sea-water of 0·074 per cent. (assuming $\chi = 0{\cdot}165$).

There was no obvious change in the retardation through the membrane and perivitelline space during the first 10 minutes after fertilization. Since, however, the main part of the retardation comes from the perivitelline space, there might have been small changes occurring in the retardation or dry thickness of the fertilization membrane itself.

Discussion

Perhaps the most interesting point in these observations is the low R.I., and therefore the low concentration, of the nuclear material. The nucleus of the unfertilized egg appears to be nearly twice as dilute as the cytoplasm, and does not change appreciably on fertilization. The chromosomes at metaphase must be somewhat more concentrated than the resting nucleus since they have the same R.I. as the spindle and the asters, but they never appear to reach the R.I. of the cytoplasm. After metaphase they become more dilute; perhaps as a result of the swelling and vesiculation of late anaphase and telophase. The low concentration of material in the nucleus and chromosomes is somewhat surprising, but it may be a situation restricted to eggs. The nuclear material in tissue culture cells, amoebae, and spit cells has a higher R.I. than the cytoplasm.

The only curious thing about the changes of cytoplasmic R.I. in interphase is that it tends to drop during mitosis. If, as seems reasonable from the centrifuged eggs, the low R.I. of the mitotic figures is due to the expulsion of granules, it might be expected that the R.I. of the surrounding cytoplasm would rise proportionally. The explanation may be that during metaphase and early anaphase, when the R.I. difference between asters and cytoplasm is greatest, the volume of the cytoplasm is much greater than that of the asters and so the increase in total R.I. is not appreciable.

Two other workers have published figures on the R.I. of developing eggs. Vlès (1921) measured the R.I. of sea-urchin eggs by the ingenious method of treating them as a lens and measuring their focal length. He found an R.I. of about 1·385 during interphase and a rise to 1·405 just before division. These values seem rather high (an R.I. of 1·405 represents a concentration of 44 per cent. with $\chi = 0{\cdot}165$), and we found no sign of such a large rise before division. Pfeiffer (1951) measured the R.I. of *Nereis* eggs and other cells by pushing a series of glass needles of varying R.I. into the cells and seeing when the needles vanished. It is hard, however, to take very seriously a method which, for one stage of a *Nereis* egg, gave an R.I. of 1·53. This is greater than the R.I. of pure gelatin (1·525).

The average figure of 25·5 per cent. of solids in the unfertilized egg seems quite reasonable, but it is somewhat higher than the 18·1 per cent. found by McClendon (1909) for the unfertilized egg of another sea urchin, *Arbacia punctulata.*

The measurements on the fertilization membrane and perivitelline space ve direct evidence of the presence of a slightly more concentrated solution an sea-water in the perivitelline space. This substance is presumably a lloid, since salts are known to penetrate the membrane. Such a substance as postulated by Loeb (1913) to explain the elevation of the membrane. The gure of 160 Å (±80 Å) for the *dry* thickness of the fertilization membrane grees quite well with the figure of 100 Å given by Mitchison (1954) from ectron microscope measurements. The hydrated membrane in its normal ate is undoubtedly thicker, about 500 μ (Mitchison 1954).

The fact that the jelly round the unfertilized egg has no detectable retarda- on means that it must contain very little solid matter. If the least detectable etardation is taken as $\lambda/300$ and the jelly diameter as 200 μ, then the jelly must ontain less than 0·0055 per cent. of material over and above sea-water $\chi = 0{\cdot}165$). This very low concentration must imply relatively enormous paces between the long-chain molecules of the jelly.

These results show that the interference microscope is likely to be a valuable ool. For the purpose of observation the relatively high sensitivity can reveal ew structure, e.g. the low R.I. patches in some unfertilized eggs, and the resence of a colloid in the perivitelline space. The interference colours in white light are also very striking and they serve not only to identify the Newtonian orders but also to differentiate very clearly between true retarda- ion changes which affect the colours, and other extraneous factors in the microscope system (e.g. irregular illumination or dirt on the lenses) which only affect the light intensity. As a measuring instrument the microscope is extremely sensitive to small changes in concentration, but it is limited in its capacity to give absolute values of concentration by the uncertainty of the χ values or refractive increments for biological material.

We should like to express our thanks to Messrs. C. Baker Ltd. of 244 High Holborn, London, for the loan of the microscope; and to the Director and Staff of the Marine Station, Millport, for their help. The work was carried out while one of the authors (J. M. M.) was at the Dept. of Zoology, University of Cambridge.

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The Structure of the Spermatozoa in Dextral and Sinistral Races of *Limnaea peregra*

By G. G. SELMAN AND C. H. WADDINGTON

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With one plate (fig. 1)

SUMMARY

Spermatozoa from dextrally and sinistrally coiled races of *Limnaea peregra* were xamined by the use of an electron microscope. Helical fibres in the middle and tail-ieces of the sperm were found to be dextrally coiled in preparations from individuals f both races. It is concluded that the genes which determine the direction of spiral eavage in *Limnaea* do not control coiling in the sperm.

The structural features of the sperm are discussed in the light of limitations imposed y geometrical considerations on the form of the helical fibres.

INTRODUCTION

THE body and shell of the well-known fresh-water snail *Limnaea peregra* are normally coiled in a right-handed spiral, that is to say, in the same irection as the ordinary carpenter's screw. Occasional races have been found with the opposite or sinistral direction of coiling. Genetical studies were made n one such race some years ago by Boycott, Diver, and their associates Boycott, Diver, and Garstang, 1925; Boycott, Diver, Garstang, and Turner, 931; Diver and Andersson-Kottö, 1938). It was shown that the direction of oiling is determined by genes which operate either in the maternal ovary or n the unreduced egg. These genes, therefore, have a delayed action, the haracter which they determine being shown not in the animal which carries hem but in the offspring of that animal. A second sinistral race has recently een studied in this laboratory by Mrs. F. M. Robertson (1953) and a similar genetic situation was found. The direction of coiling of the animal can be recognized in the very early cleavage divisions of the egg, in which the first blastomeres are arranged either in the right- or left-handed spiral form.

This situation has attracted a good deal of attention not only because it provides a clear-cut example of the control of egg structure by genes in the maternal organism, but also because we have here an instance of a gene which is in very close causal connexion with a structural as opposed to a chemical property of the organism. We have as yet little understanding of the physico-chemical mechanisms by which animal structure is produced. Although the *Limnaea* egg presents one of the most clear-cut examples of the genetic con-trol of such a character, we have little idea of the means by which the genes may act. Alterations in the time relations between the process of cell division

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and the processes, whatever they are, which orientate the mitotic spindl might be held responsible for the difference between a left-handed and right-handed spiral. Another hypothesis which might at first sight seem m attractive would be that the gene participates directly in the determinati of the stereo-chemical form of certain structural proteins in the egg cytoplas and that the direction of cleavage is determined by this ultra-structure of t cell (Needham, 1936, 1942).

Confirmation of the latter suggestion would be obtained if it could shown that other structural features of the dextral and sinistral races unrelate to the egg structure were also affected in a similar way by the egg-determinin genes. *Limnaea* belongs to the sub-class Euthyneura, and the spermatozoa these forms have long been recognized from studies with the light microscop (Leydig, 1883; Platner, 1885; Retzius, 1906) as having tail and mid-piec formed of two or more elements twisted round each other to give long helice reminiscent of the form of twisted strings. As the wide comparative studie of Retzius (1906) showed, this is not the structure in the sperms of th Streptoneura. Although the electron-microscope has revealed or confirme helical micro-structure in tail and mid-pieces of sperms from animals c diverse groups (Bretschneider, 1949; Randall and Friedlaender, 1950; Harr son, Randall, and Bayley, 1952), yet among the molluscs, the helical coil i more obviously impressed upon the external form in the Euthyneura than i any other group. Indeed outside the Euthyneura, the only sperms with comparable structure known to us are those of passerine birds (Retzius, 1906 Ballowitz, 1913). It therefore seemed worth while to examine the sperm o *Limnaea* in more detail to find out if the direction of coiling was affected b the genes which control egg spiralization.

MATERIAL AND METHODS

Mature sperms from individuals of dextral and sinistral races of *Limnae peregra* were examined under the electron microscope. To decide the directio of coiling it is not sufficient to use a light microscope. This is confirmed by the fact that Platner (1885) and Retzius (1906), after examining sperms from *Limnaea stagnalis*, came to opposite conclusions concerning the form of the helices. An electron micrograph shows the direction of spiralization clearly, and confirmation may be obtained by using the shadow-cast technique.

The dextral and sinistral lines of *Limnaea peregra* were taken from stocks, maintained in this laboratory, whose genetic history was known. Each testis was dissected into one-tenth strength Holtfreter's saline. The sperms were released into about 2 c.c. of a similar solution or into distilled water. A minute drop of the resulting suspension was placed upon each electron-microscope grid, previously filmed with celloidin. Fixation was then achieved by exposure to the vapour of 2 per cent. osmium tetroxide for 2 minutes (Porter and Kallman, 1953), except for a few specimens which were air dried. After fixation specimens were dried in a vacuum desiccator, and subsequently washed in two changes of distilled water and dried again. About half the specimens were shadowed at an angle of 25 degrees (or in some cases 40 degrees) to the plane of the grid with palladium-gold alloy. The specimens were examined in a Metropolitan Vickers E.M. 3 electron-microscope operated at 75 kV.

Certain of the specimens had been pre-treated with ultrasonics at an intensity of 7 watts/cm.^{2} for 2 to 5 minutes, in order that the sperms might be partially broken down into their structural units. The ultrasonic apparatus used was similar to that described by Selman and Wilkins (1949).

Although most of the structural details described here were based upon the study of electron-microscope photographs, they were supported by examination of living sperms under the phase-contrast and polarizing microscopes, and of fixed and stained smears under the light microscope.

THE STRUCTURE OF THE SPERMATOZOON OF *LIMNAEA*

In broad outline, the spermatozoon consists of a small, roughly conical head-piece, a mid-piece, and a long tail ending in a terminal filament. The total length is rather more than a quarter of a millimeter.

The Feulgen-positive head is 3·2μ in length, approximately circular in section and opaque to the electron beam. From a rounded tip it widens gradually to a maximum diameter of just under 1·2μ, about 0·6μ from the base. The basal diameter is approximately 1μ. The head, however, is not a perfect cone and is slightly spiral in form. The helical fibres of the mid-piece appear to pass over the neck region to form an outer envelope for the head. Thus obscured, the neck region appears simply as a constriction 0·6μ in diameter, beneath a spiral covering (fig. 1, A).

Live sperms were examined with the polarizing microscope. The anterior three-quarters of the sperm head were negatively birefringent with respect to the long axis of the sperm, while the base of the head was, like the mid-piece, positively birefringent. It is probable therefore that, in the anterior region of the head at least, the nucleic acid columns are packed parallel to the long axis of the sperm (cf. Schmidt, 1937).

The mid-piece extends posteriorly for about 40μ from the neck. It is apparently more rigid than the main tail-piece since it is less commonly found with a sharp bend in it when a smear preparation is examined. Measurements on electron micrographs show that the outer sheath consists of seven fibres, each about 0·1μ in diameter and of elliptical or approximately circular section, wound evenly upon a core approximately 0·5μ in diameter near the head and 0·4μ at the posterior end (fig. 1, D). Studies of spermatid development (Platner, 1885; Wilson, 1928) make it probable that this core is the axial filament which continues down the axis of the main tail-piece to become the terminal filament. Each fibre of the sheath forms a helical coil of pitch 3μ near the head and 4μ at the posterior end of the mid-piece.

The main tail-piece is approximately 230μ in length. It also consists of helical fibres wound upon the axial filament. The helical fibres are larger and more prominent at their anterior ends; as the terminal filament is approached not only do they become smaller in diameter but they lie much flatter and are less easily seen. At the anterior end four fibres can be distinguished; two of them, which lie together, are rather more than 0·2μ in diameter, a third is approximately 0·15μ in diameter, while alongside it is a fourth which is slightly less than 0·1μ in diameter (fig. 1, B). The pitch of the helical coils is about 4μ anteriorly, and is 5μ towards the posterior end. The tail-piece tapers from an outer diameter approximately 0·7μ at the anterior end to about 0·4μ at the terminal filament.

Surprisingly, there is no obvious discontinuity to mark the joint between the middle and tail-pieces. Tracing the course of individual helical fibres over the section concerned, one is led to the conclusion that six fibres of the mid-piece coil join together in pairs to give the three larger fibres of the tail-piece, while the seventh fibre of the mid-piece is continuous with the small fourth tail fibre. The appearance, pitches, and diameters of the fibres agree with this interpretation. Naturally the helices of the mid-piece and tail-piece are wound in the same direction.

It was found that all the coils were dextral (as in a carpenter's screw), no matter whether they were from testes of dextral or sinistral snails. (This conclusion is actually based upon the examination of electron micrographs of twelve sperms definitely with dextral coils from snails of sinistral genotype, and eight sperms definitely with dextral coils from dextral snails.) Hence, although the sinistral allele when homozygous determines in the oocyte cytoplasm the sense of the spiral pattern to be followed during subsequent cleavage and shell formation, it does not determine the sense of the spiral pattern followed in the formation of the sperm.

The terminal filament is 20μ long, approximately 0·16μ in diameter, and is easily separated into ten fibrils each about 30 mμ in diameter (fig. 1, E). Thus the terminal filament might appear whole or broken into fibrils in adjacent sperm of a preparation which had been given no special treatment.

FIG. 1 (plate). In each figure the scale represents 1 μ. An arrow indicates the direction of shadowing.

A, head, neck, and anterior part of mid-piece. Dextral snail. Shadowed at 40°. Fixed in the vapour of osmium tetroxide.

B, anterior part of tail-piece (flattened). Helical fibres: A and B, 0·2μ diameter; C, 0·15μ diameter; D, 0·07μ diameter. Dextral snail. Shadowed at 40°. Fixed by air drying.

C, fragmented fibre showing fibrils with helicoid form. Sinistral snail. Treated with ultrasonics at 7 watts/cm.^{2} for 2 minutes. Fixed in the vapour of osmium tetroxide.

D, part of mid-piece. Underlying fibrils are from terminal filament of another sperm. Sinistral snail. Fixed in the vapour of osmium tetroxide.

E, posterior part of terminal filament showing fibrils. Shadowed at 25°. Fixed in the vapour of osmium tetroxide.

F, posterior part of tail-piece broken to show axial filament. Treated with ultrasonics at 7 watts/cm.^{2} for 3 minutes. Shadowed at 25°. Fixed by air drying.

G, fibrillar remnants from sperm after treatment with ultrasonics at 8 watts/cm.^{2} for 5 minutes. Shadowed at 25°. Fixed by air drying.

I, head, neck, and anterior part of mid-piece. Dextral snail. Fixed in the vapour of osmium tetroxide.

J, tail-pieces of two sperms. Note that the horizontal tail-piece, which is from a more posterior region, has coils of greater pitch. Sinistral snail. Fixed in the vapour of osmium tetroxide.

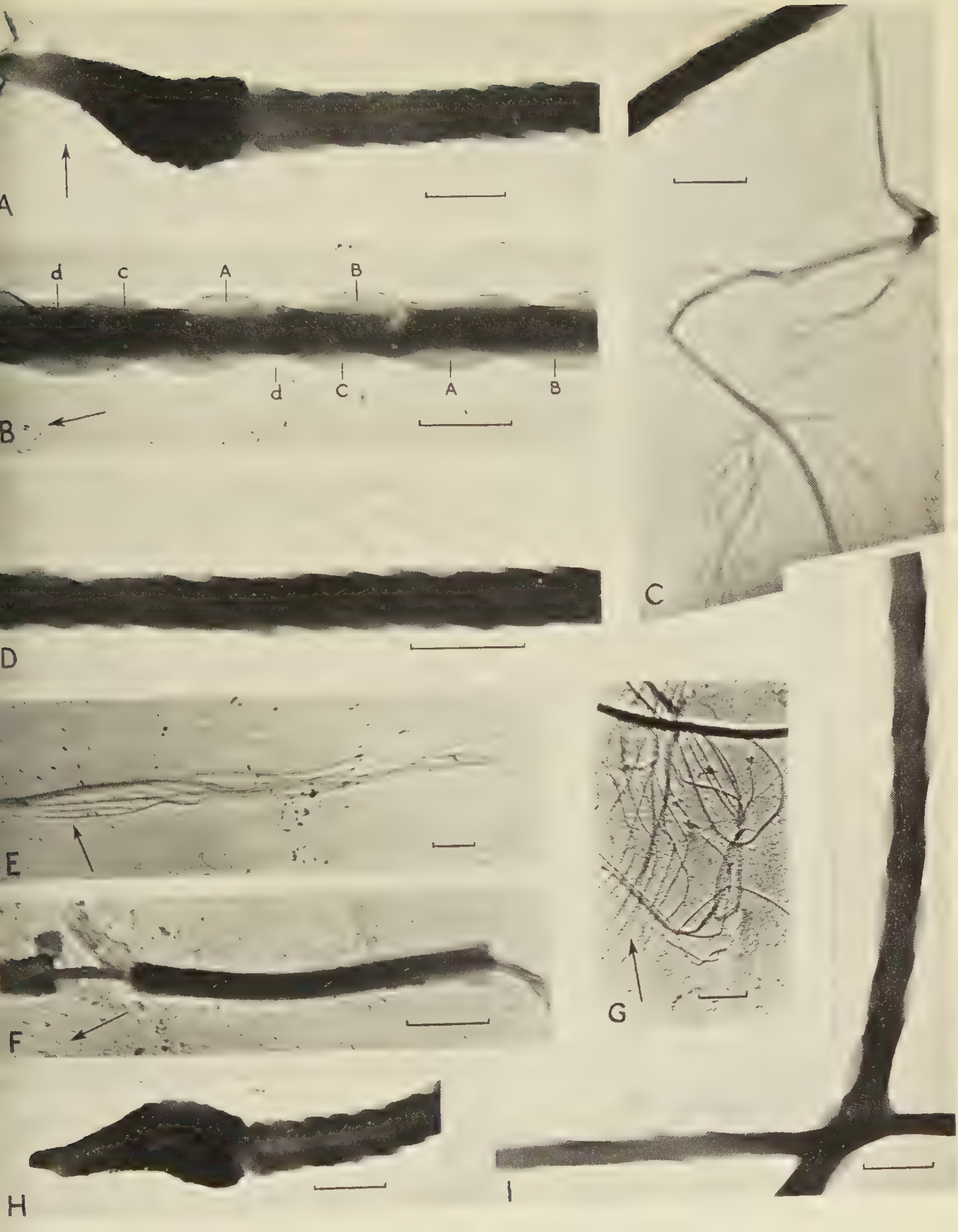

Fig. 1

G. G. SELMAN and C. H. WADDINGTON

In preparations which had been treated with ultrasonics it was possible to find sperms partially disrupted into their fibrous components and with the fibres themselves unravelled into constituent fibrils. Although micrographs of such disruptions were mostly difficult to interpret by reason of the complexity revealed, a favourable instance (fig. 1, C) shows a tail-piece fibre from the helical sheath resolved into ten fibrils, each about 25 mμ in diameter. These fibrils appear to have been packed in a helicoid manner in the fibre, but we have been unable to determine the direction of coiling at this level of organization. Another photograph (fig. 1, F) shows a posterior section of tail-piece broken so as to expose the central axial filament. If the ultrasonic treatment is sufficiently severe the sperms are completely broken down into a mass of long fibrils each approximately 20 mμ to 30 mμ in diameter (fig. 1, G).

DISCUSSION

It is clear from the above that the genes which determine the direction of spiralization of the egg cleavages and the resulting snail do not have a similar influence on the direction of coiling of the sperm. One must conclude that if the genes determine the spiralization of the cleavage by an influence on the stereo-chemical nature of the protein constituents of the cytoplasm, then these proteins play a relatively small part in the formation of the spermatozom and are unable to determine its coiling. It appears more probable that the genes act on the spiralization of cleavage by some other mechanism which does not directly involve the structure of the cytoplasmic proteins.

The middle and tail-pieces of the sperm have been shown to consist of a number of fibres wound in helical coils so as to cover an axial core which tapers gradually from 0·5 μ near the neck to about 0·2 μ near the terminal filament. It may seem surprising that a sheath provided by seven helical fibres in the mid-piece should be replaced by a lesser number of fibres in the tail-piece, but this and other structural features may be explained by considering the geometrical properties of a model shown in fig. 2.

With reference to the symbols used in the diagram it can be shown for such a coil that

$$\frac{d}{t} = \frac{Nk \sec\theta}{\pi} - 1$$

This equation also applies to fibres of circular section with radius t, which do not touch each other but are separated by a distance $(k-1)t$. It also holds for fibres which have shrunk through fixation to such a state, or to a combination of all three circumstances; the exact shape of the fibre section is not critical.

From the equation it is clear by inspection that with N, k, and t constants, as d is reduced it is necessary to reduce $\sec\theta$ also. As the core decreases in diameter, therefore, the pitch of the fibres increases. This is observed in the mid-piece and particularly in the tail-piece (fig. 1, I). But $\sec\theta$ cannot assume real values less than unity, and a critical diameter is reached when, in order to accommodate N fibres with particular values for k and t, θ must be zero and the fibres are each parallel to the axis of the sperm. It is impossible to pack such fibres round a core of diameter less than the critical value without reducing their number.

For the case of the mid-piece there are seven fibres, and taking values of 0·45 μ for d, 2·3 μ for k, and 0·1 μ for t, a value for θ of 21 degrees is obtained which agrees with observation. Retaining these values for k and t, and putting θ at zero, we obtain for seven such fibres a critical value for d of 0·41 μ. This value for the core is actually found near the join between the mid-piece and the tail-piece. As the core diameter of 0·41 μ is approached the helical sheath of seven fibres would have to be replaced by a lesser number of larger fibres if they were still to fit themselves round the core. Actually this change occurs before the limiting conditions have been reached.

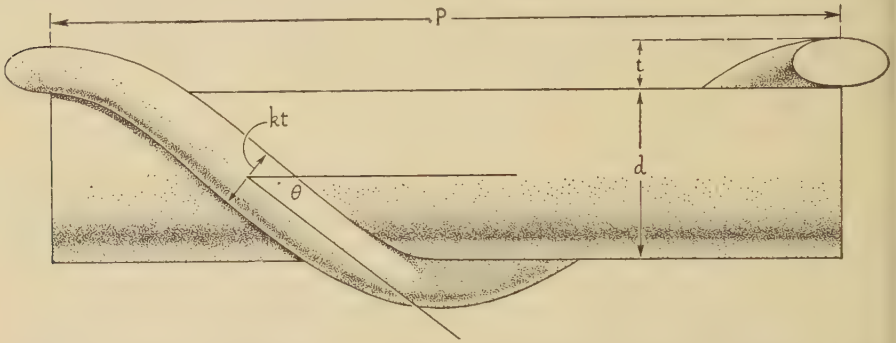

FIG. 2. The diagram shows one of N helical fibres, elliptical in section, of major and minor axes kt and t respectively, wound with their minor axes in contact with a cylindrical core of diameter d so as to cover it. Each fibre is of pitch P, and at all points along its length it makes an angle θ with the axis of the sperm.

In the anterior region of the tail-piece, four helical fibres are observed with values for t of 0·2 μ, 0·2 μ, 0·15 μ, and 0·07 μ. To a first approximation these may for mathematical simplicity be replaced by considering three fibres each of diameter 0·2 μ. Then using the equation again with values of 20 degrees for θ, and 2·3 for k, it is found that three such fibres are wound upon a core of 0·27 μ diameter. It is preferable, however, to use the actual observed values for the diameters of each of the four fibres, and to substitute them in a suitably modified equation. With the same values for θ and k, we then obtain a core diameter of 0·33 μ which is in very good agreement with observation. The approximate formula gives better agreement at a more posterior region of the tail-piece where the small fourth fibre is difficult to distinguish. Towards the posterior end of the tail-piece the fibres themselves taper to a value for t which approaches 0·14 μ. With this value for t, and again with 2·3 for k, we may obtain from the equation a critical core diameter for three fibres of 0·17 μ. This is in such good agreement with the observed value for the axial filament diameter at the extreme end of the tail-piece that one is tempted to conclude that these geometrical considerations may determine the point at which the tail-piece ends abruptly leaving the axial filament exposed.

This paper is dedicated to Professor F. Baltzer as a tribute to him on his seventieth birthday.

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Intracellular Membranes

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With two plates (figs. 1 and 2)

Summary

Spheres that stain with neutral red in cephalopod neurons show changes with variations in the tonicity of the surrounding medium. In hypertonic solutions the surface becomes shrunken and the stainable matter restricted to crescents or granules at the side of the sphere. These processes are reversible. They may be interpreted by considering that the surface of the spheres consists of material with properties partly analogous to those of a semi-permeable layer and partly to a membrane, that is to say a layer with tangential cohesive forces. These analogies are not perfect, however, and in particular the cohesive properties of the 'membrane' are limited and its material tends to flow together to form drops when the inner pressure is reduced.

The appearances produced by the classical methods for staining the Golgi apparatus are a result of distortions of these droplets similar to those produced by changes in tonicity. The outer more osmiophil or argentophil component adopts forms reminiscent of those shown by the component that stains more deeply with neutral red.

NO living organism consists of an optically homogeneous mass and the presence of the subdivision that the microscope reveals is no doubt one of the features that allows living systems to perform their characteristic activities. The efficient use of energy for the maintenance of a steady state and the extraordinary feats of synthesis at a relatively low temperature may depend largely upon the separation of the material into compartments within which accumulation can occur. These divisions are of orders of size ranging from that of the nucleus downwards to the molecular level. Optical and electron optical methods are essential to provide the resolution necessary for the study of an organization at such a small scale. The difficulty is to interpret by means of appropriate models the images of objects that are too small to be examined directly by mechanical, electrical, or ordinary chemical methods. The framing of proper experiments for elucidation of intracellular images is therefore of crucial importance for cytology. The relatively slow progress of the science has perhaps been partly due to lack of consideration of the implications and limitations imposed by the models used in description.

This is well illustrated by the history of the Golgi 'apparatus'. Many terms have been employed to describe it and various suggestions put forward in attempts to relate its properties to those of model systems. After more than fifty years of study, however, there are remarkably few useful things that can

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be said about this material, beyond that it occurs in all or nearly all animal cells and in many if not all cells of plants.

If the significance of these bodies is that they form isolated compartments within the cytoplasm, it is obviously of importance to have an understanding of the nature of the surfaces by which they are bounded. Certain observations on the neurons of cephalopods suggested that the 'Golgi apparatus' consists of spherical bodies provided with a semi-permeable surface membrane (Young, 1932). Various authors have shown that classical silver and osmium techniques reveal in the nerve-cells of molluscs, as in so many other tissues, that the Golgi material includes a more central chromophobe and an outer argentophil or osmiophil component (Legendre, 1909; Kolatchev, 1916; Kunze, 1921; Brambell and Gatenby, 1924; Boyle, 1937). Parat in 1928 found that after staining neurons of various molluscs with neutral red a number of round bodies can be seen in the region around the nucleus, and he identified these with the Golgi material. He applied his analogy with the vacuole of plant cells, calling the material the vacuome, consisting of a more watery phase within the cytoplasm. An implication of this view might be that these bodies are provided with semi-permeable surface membranes, and evidence of osmotic effects was provided by examination of the neurons in solutions of varying tonicity (Young, 1932). In hypertonic solution the material stained with neutral red showed appearances suggesting a shrinking away from the surface of the 'vacuole'. Worley (1944) later also reported that hypertonic solutions caused shrinkage of the Golgi bodies and suggested that this shrinkage was responsible for many of the classical Golgi figures.

The importance of this and other implications of Parat's model have become increasingly apparent in recent years with the growth of evidence for the existence of intracellular membranes able to separate phases of distinct ionic composition. Observations on mitochondria by biochemical, optical, and electron optical methods have suggested the presence of a surface layer distinct from the interior (Claude and Fullam, 1945; Hogeboom, Schneider, and Pallade, 1948; Schneider and Hogeboom, 1951; Harman, 1950; Mühlethaler, Müller, and Zollinger, 1950; Spector, 1953). Mitochondrial fractions isolated from kidney and other tissues show powers of osmotic regulation and capacity to operate a sodium pump mechanism (Bartley, Davies, and Krebs, 1954).

Cytologists have been unwilling, however, to use the model of a system of 'vacuoles' within the cell because of certain over-simplifications made by Parat in attempting to describe the Golgi apparatus in terms of a single component, the vacuome, together with activated mitochondria ('chondriome actif'). There are undoubtedly several phases in the Golgi complex and they do not all stain similarly with the classical methods. This may be illustrated in the case of cephalopod neurons. After fixation in a mixture of chromic acid, potassium dichromate, and osmium tetroxide made up with sea-water, and further treatment with osmium tetroxide (Kolatchev's technique), the region around the nucleus contains a series of approximately spherical bodies (fig. 1, A–D). These take up the osmium to varying extents and in many cases small darkly staining granules are seen attached to larger lightly staining spheres. If the section is counterstained with acid fuchsin the osmiophobe component of the spheres becomes red, and large spheres towards the periphery of the cell, only faintly visible without the red stain, become conspicuous.

A cell fixed in formalin and cobalt nitrate and stained with silver according to da Fano's technique shows similar appearances. If the stain is dark there will be a series of spheres with irregular surfaces around the nucleus (fig. 1, E and F). If the stain is fainter the spheres will be seen to have pale centres with irregular rims of darker granules (G). In some cases the larger spheres appear to have irregular crenated surfaces (H and I). Sometimes the dark material appears at the side of flattened oval bodies (J), and in extreme cases the individual droplets are hardly recognizable and the area around the nucleus is occupied by an irregular meshwork of dark strands.

We can say, then, that the region around the nucleus is seen in fixed preparations to be occupied by a large number of spherical bodies of various sizes, and not all of identical staining properties. Around the outer surfaces of these and between them is much osmiophil and argentophil material. This may take the form either of granules or of crescents or rods attached to a less-stainable component.

All of these appearances are well known in neurons and in other types of cell (Baker, 1944; Thomas, 1947). The difficulty is to decide the best model for interpretation of the results of these empirical 'Golgi apparatus methods', which involve prolonged treatment with various chemical mixtures. The difficulty is reflected in the multiplicity of terms that have been applied to this material. There is a general agreement as to the existence of two phases (internum and externum, &c.), and it is often suggested that the more osmiophil component makes a sheath or membrane around the less-stainable centre. In the fixed preparations the dark and light materials appear in various relations to each other. As will be shown later, changes in the tonicity can produce drastic but reversible alterations in the appearance of these materials as seen after staining by neutral red. How much greater change must we expect from fixation in the complex mixtures used in the classical techniques?

Neutral red sometimes stains bodies already existing in a cell, but in other cases it becomes segregated in newly formed 'vacuoles'. In cephalopod nerve-cells the bodies can be seen in the unstained state without application of neutral red and they are not products of its action. They can conveniently be studied by making hand sections, as thin as possible, of the stellate ganglion of octopus. If these are placed in a solution of about 1/50,000 neutral red in sea-water, then the bodies will begin to be coloured in about ten minutes, and a maximal stain is reached after half an hour. The larger and more peripheral bodies stain first, and when the stain has fully developed there is a dense mass of granules around the nucleus, of all sizes from about 5μ to 0·5μ or less.

In sea-water (which is isotonic with the blood, though differing in its ionic content, Robertson, 1953), the bodies are often not simple spheres. The larger ones may have the crenated appearance seen in fig. 2, A and C, and in the medium and smaller ones the darkly red material is often in the form of crescents at one side of the droplet, whose outline is, however, faintly defined.

If the sections are stained in a solution of the same strength of neutral red but in sea-water diluted with an equal volume of distilled water, all the bodies have the form of spheres (fig. 2, B and D). This effect of a hypotonic medium at once suggests that processes analogous to osmosis may be at work, producing shrinkage even in the 'isotonic' solution. This was confirmed by a series of experiments in which a section was stained in a solution of neutral red made up in sea-water and a diluted solution was then drawn under the coverslip. Under favourable circumstances it was possible to photograph the same cell under the two conditions. Bodies that were shrunken and crenated in the stronger solution could be seen to become fully rounded in the more dilute one. The red material changed from the form of a crescent at the side of a drop to a uniform distribution in the weaker solution.

These results can be extended by staining sections in hypertonic solutions, containing twice or three times the normal concentration of salt. Under these conditions extreme forms of concentration of the stain into granules were often seen (fig. 2, F, I, and J). The coloured matter appeared either as a dense crescent at one side of the body, or as one or more granules at the extreme edge, looking in some cases as if it lay outside the main droplet. These conditions can also be reversed by treatment with more dilute solutions, in which the bodies become simple spheres.

In the fixed preparations the droplets seen may be round but they also often show irregularities similar to those produced by hypertonic solutions acting upon the neutral red bodies. The irregular bodies of fig. 1, G–I, correspond closely to the shrunken droplets of fig. 2, A and C. The crescent-shaped 'dictyosomes' of fig. 1, J, resemble the 'vacuoles' in which the red stain occupies one side only (fig. 2, C). It is tempting to regard the droplets of osmiophil material seen around the osmiophobe bodies as comparable with the deeply stained droplets of neutral red seen in hypertonic solutions, or at least to consider that the two appearances are somehow related. The component that stains darkly in fixed preparations may have a high phosphatide content (Baker, 1944) but there is no obvious reason why neutral red should stain such material. The osmiophil material is evidently present in considerable concentration at the surfaces of these bodies and as a result of the procedures

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FIG. 1. Photomicrographs of neurons of *Octopus vulgaris*. E and F are from the sub-oesophageal ganglia, the rest from the stellate ganglia. The scale at the bottom applies to all the photomicrographs.

A–D, Kolatchev technique, showing lightly staining spheres to which are attached more deeply impregnated osmiophil granules or rims. A is counterstained with acid fuchsin, which has coloured the less osmiophil droplets red.

E–J, da Fano's technique, with a light counterstain of Ehrlich's haemotoxylin in E, F, I, and J.

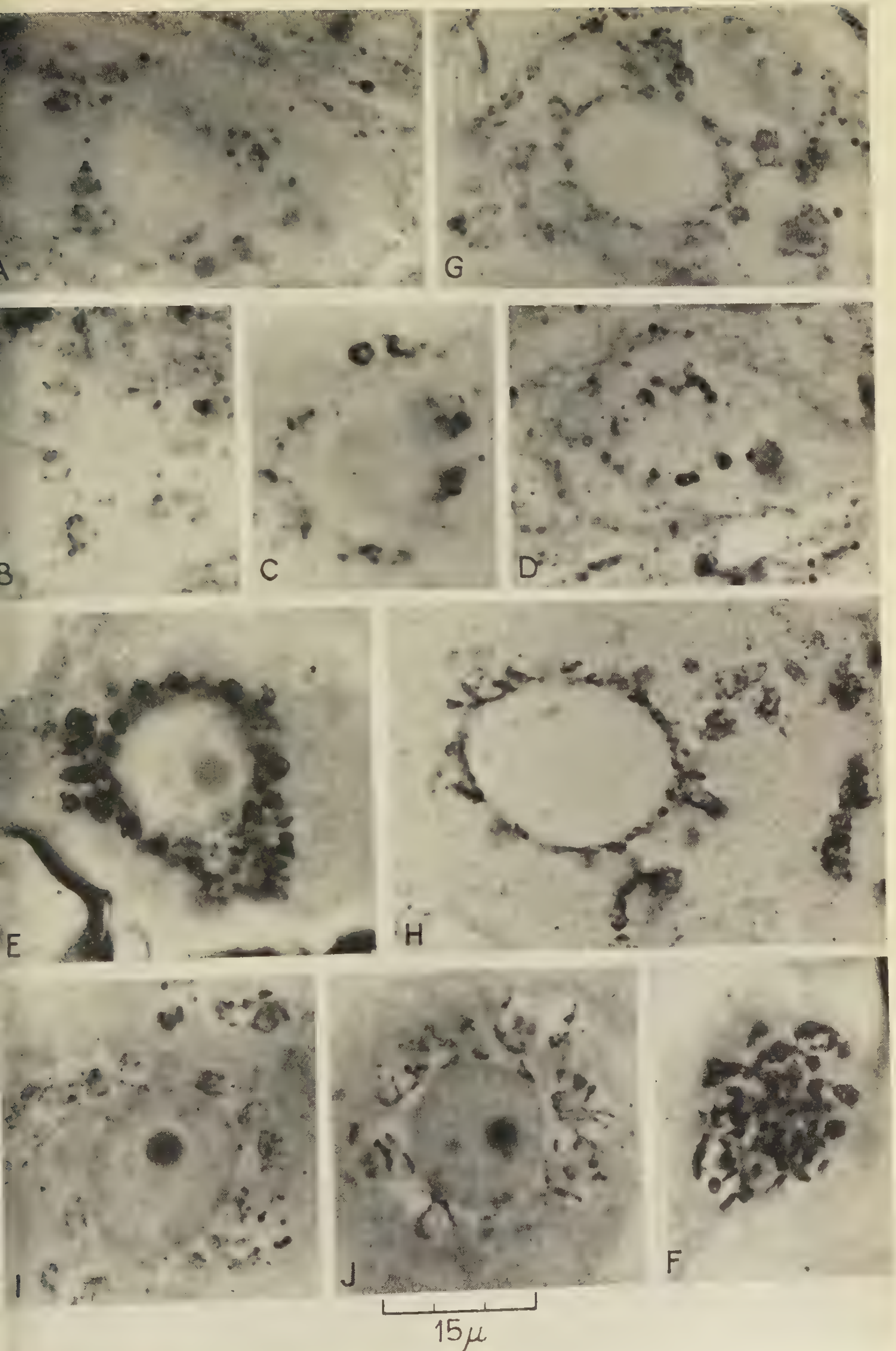

FIG. 1

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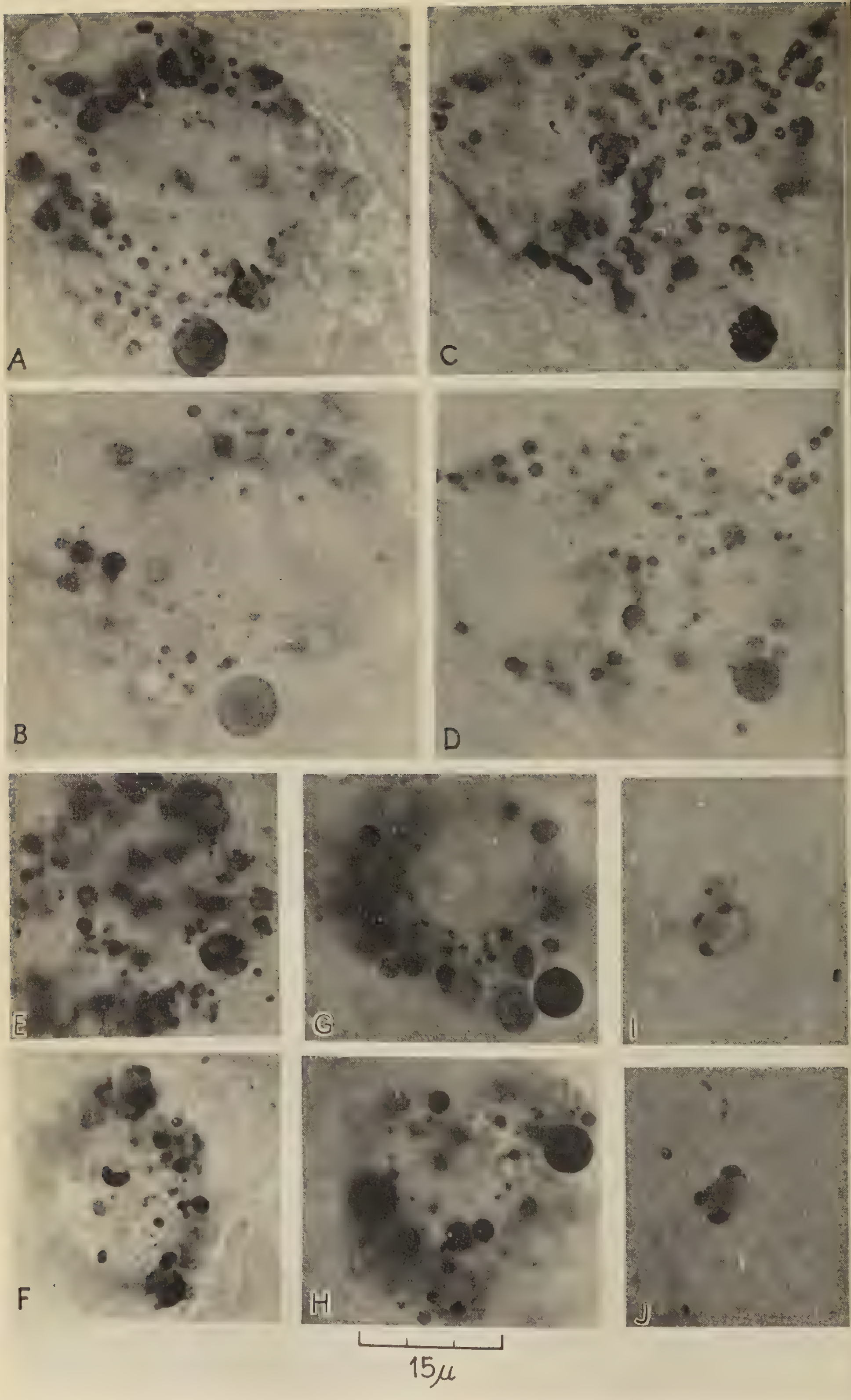

FIG. 2

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adopted in the classical techniques it may become segregated or disaggregated from the rest of the drop, producing the various well-known forms of 'network'. Hirsch (1939) has suggested that this change resembles that seen between the phases of a co-acervate. Whatever the nature of this stainable material may be it seems likely that its presence serves to separate phases inside and outside the drop, perhaps in such a manner as to assist in specific enzymatic activities, if these are characteristic of the Golgi material (see Baker, 1944).

Little is known of the activity of these perinuclear droplets in cephalopods. Treatment with the 'Nadi' reagent shows that the products of indophenol-oxidase accumulate in this region (Young, 1932), but this may of course be a result of concentration here of the reagent or product. There is much evidence for the localization of enzymatic properties in mitochondria and Golgi bodies and the presence of special layers around their surfaces therefore assumes special significance. Janus green stains material at the outer rim of the droplets that are coloured with neutral red, and there may be some relationship between the outer material of the Golgi complex and that of the mitochondria.

The relation of the Golgi bodies to the mitochondria is as obscure in these neurons as in other cells. The rodlets seen in the perinuclear region may resemble mitochondria, but classical cytological techniques show stained rodlets distributed throughout the cell body (Young, 1932). There are resemblances as well as differences between the two types of cell component. Harman (1950) has shown that in hypertonic solutions mitochondria assume a crescent shape similar to that reported here. Enzymatic activity was greater in the spherical condition.

Such changes produced in the neutral red bodies or mitochondria by variation in the medium suggest that their surfaces can be considered by comparison with a semi-permeable membrane as a model. The shrinkage and crenation seen in fig. 2, A and C, must be due to the withdrawal of water through a membrane that has sufficient stability to remain as a crumpled sheet, and can be distended again in a more dilute environment. However, the application of this model to the other appearances raises difficulties. The crescent-like appearances might be interpreted as due to the withdrawal of

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FIG. 2. Photomicrographs of neurons from the stellate ganglia of *Octopus vulgaris*: hand-cut sections stained with neutral red about 1/50,000.

A and C are cells that have been for 30 minutes in neutral red in sea-water. Neutral red in sea-water diluted with an equal volume of distilled water was then drawn under the coverslip. B and D are pictures taken 20 minutes later of the cells seen in A and C.

E and F are from pieces that have been stained for 30 minutes in neutral red in sea-water to which 3·5 per cent. of sodium chloride has been added. A solution made up in sea-water diluted with an equal volume of distilled water was then drawn under the coverslip and the pictures G and H were taken 30 minutes later. They are of cells very close to those shown in E and F.

I and J, granules in cells stained for 30 minutes in neutral red to which 3·5 per cent. of sodium chloride has been added.

water from an inner compartment *within the droplet*. Since the outline of the whole body remains intact one would then be forced to the conclusion that there are *two* membranes around each body and that the outer one maintains approximately the spherical form when the inner volume is reduced by plasmolysis. If this was so we should have to decide what occupies the more lightly stained region of the droplet. The separation of the more darkly stained material might alternatively be regarded as a precipitation or some other form of disaggregation of a phase within the droplet, but it is difficult to see why this should produce the crescent forms that so often appear. The crescents are not always similarly orientated in a given part of the cell and the effect is not due to the sweep of a wave-front of differing conditions.

Neither the simple plasmolysis interpretation nor that of precipitation seems adequate. A third alternative is that the darkly stained material may perhaps be considered as a shrunken surface layer or cap, rather than as an internal compartment. This would imply that the stable portion, which remains spherical in hypertonic solutions, is inside the more darkly staining material.

This would also explain the highly coloured, small granules seen at the surface of the bodies in very hypertonic solutions, fig. 2, I and J, which are still more difficult to interpret in terms of a simple semi-permeable model. It is difficult to believe that they could be due to shrinkage of an inner component that has a membrane-like covering and can lose water independently of the rest. It is possible, though perhaps unlikely, that in a hypertonic medium the neutral red stains bodies not seen in more dilute solutions. Thomas (1947) reported that neutral red injected into the haemocoele of snails stained small dark granules attached to the 'spherical complexes'. Only further experiment can show the nature of these dark-staining masses, but one interpretation is that they represent an extreme shrinkage of an outer component, earlier stages of which are seen in the collapse of the larger droplets and the formation of crescents.

We need in any case to adopt something more subtle than the simple model of a semi-permeable membrane. Concepts of membranes and their action, which are defined and understood on the macroscopic level, cannot easily be transferred to minute dimensions. By a membrane we understand a layer whose molecules not only act as a barrier to diffusion but also have cohesive properties, which enable a single sheet to be maintained under various physical strains. It may be that in order to obtain further insight into these processes on a small scale we need to separate the notion of a barrier from that of a sheet. The layer around the larger droplets certainly has tangential cohesive properties, for it can become folded. But we do not need to think of the folding entirely in terms of a sheet that crumples and maintains its area. When pressure is withdrawn from within, the molecules of the membrane may slide upon each other and cease to be alined regularly. There are suggestions in fig. 2, A and C, that the surface is forming aggregations at intervals, giving a mulberry-like appearance. The granules seen upon the droplets in very hypertonic solutions (fig. 2, I and J) may then be regarded as the membrane rolled off the droplet, and the crescents would be an intermediate condition.

It is difficult to speculate what effect such changes would have upon the semi-permeability of the droplet or how such an explanation could be combined with that of plasmolysis to explain the reversibility of the process. In any case it is necessary to apply the concept of semi-permeability with caution since it has been worked out fully only for membranes of larger size presumed to have a simple porous structure. The enzymatic systems by which the cell segregates materials and maintains its homeostasis are unlikely to be entirely comparable to the surfaces of porous pots. This illustrates the difficulty in the study of the finer details of cell organization that arises from the fact that we can only try to describe the appearances and experiments in terms of other phenomena that are more fully understood, even though we realize that the comparisons are incomplete, and need to be combined with others. Only in this way can cytology gradually reach sound concepts with which cellular organization can be adequately described.

The idea of intra-cellular surface boundaries is certainly one such concept, fundamental to understanding of cell organization (see Peters, 1930). However, the very elementary experiments reported here show that even the conception of a compartment is much less simple than it appears. The evidence is sufficient to prove that the mitochondria and Golgi bodies resemble each other in being bounded by membranes that have properties in some ways comparable to semi-permeability; however, this statement must be considered with the qualifications discussed above about the conceptions of 'membrane' and 'semi-permeability'.

Perhaps all that we should say at present is that all cells contain bodies of several sorts (mitochondria and Golgi bodies), each covered by an outer layer of material that confers properties comparable to semi-permeability and at least in some cases allows active ion-transport against concentration gradients. There is also abundant evidence that many synthetic activities are performed in relation to these intra-cellular bodies and the discovery of a suitable model to represent their surfaces may well be an important step in the understanding of enzyme action.

This work was done at the Stazione Zoologica at Naples, and I am very grateful for the help received there from the Director, Dr. R. Dohrn, and his staff. I am especially indebted to Mr. Angelo Sessa for his help with photographs in Naples and to Mr. J. Armstrong for similar help in London. The work was assisted by a grant from the Nuffield Foundation.

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The Cell-theory: a Restatement, History, and Critique

Part IV. The Multiplication of Cells

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SUMMARY

In the first half of the nineteenth century it was commonly supposed that new cells arose either *exogenously*, outside pre-existing cells, or *endogenously*, from small rudiments that appeared within pre-existing cells and gradually grew larger. The theory of exogeny had been founded by Wolff (1759), and was supported especially by Link (1807), Schwann (1839), and Vogt (1842). The theory of endogeny, which had been hinted at by various writers in early times, obtained the backing of a very large literature. Its chief advocates were Raspail (1825, &c.), Turpin (1827, &c.), Schleiden (1838), Kölliker (1843–4), and Goodsir (1845).

That cells do not arise exogenously or endogenously, but are produced by the division of pre-existing cells, was at last realized by the convergence of studies made in three separate fields, as follows:

(1) Trembley (1746, &c.), Morren (1830, 1836), Ehrenberg (1830, 1832, 1838), and others noticed how protists multiply.

(2) Dumortier (1832), Mohl (1837), and Meyen (1838) watched the partitioning of the cells of filamentous algae.

(3) Several observers studied the cleavage of eggs and at last revealed that this was a process of cell-division (Prévost and Dumas (1824), von Siebold (1837), Barry (1839), Reichert (1840), Bagge (1841), Bergmann (1841–2)).

Nägeli (1844, 1846) also made an important study of cell-division in all the main groups of plants (except bacteria), but used an unfortunate nomenclature that tended to obscure the true nature of the process.

Remak (1852 and 1855) and Virchow (1852, 1855, 1859) made general statements to the effect that division is the standard method by which cells multiply. The writings of Remak on this subject were much more weighty than those of Virchow.

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INTRODUCTION

We are concerned here with the proposition that *cells always arise, directly or indirectly, from pre-existent cells, usually by binary fission*; that is to say, with Proposition III in the formulation of the cell-theory adopted in this series of papers.

With a few exceptions the early cytologists appear not to have been very inquisitive about the way in which cellular structure developed: they were content to describe what they saw at a particular moment in time. About the beginning of the nineteenth century, however, attention began to be focused on the subject of the multiplication of cells. Unfortunately several false theories were promulgated at that time and gained a good deal of acceptance, so that when the truth began to be disclosed towards the middle of the century, by the convergence of unconnected studies, the new discoveries had to contend against firmly established errors. To give a realistic history of the discovery of the actual method by which cells multiply, it is necessary at the outset to present a rather full account of the erroneous views, which were expounded by such distinguished investigators as Wolff, Sprengel, L. C. Treviranus, Raspail, Schleiden, Schwann, and Kölliker. There is a special reason why the exact nature of the errors should be understood. As we have already seen in this series of papers, it happens from time to time that someone alights casually on a particular passage in an old book or journal and attributes a discovery to the author of it, when critical reading and thorough preliminary knowledge would have shown that the writer of the passage actually held entirely mistaken opinions. A careful history of such opinions is necessary if credit is to be restricted to those who really deserve it.

A study of the very extensive literature of the subject reveals that there are three main methods by which cells have been supposed to multiply. These will here be called *exogeny*, *endogeny*, and *division*. By exogeny I mean the origin of new cells outside existing ones; by endogeny, the growth of new cells from small rudiments within an existing cell; and by division, the carving up of an existing cell into two or more smaller ones.

The following classification of the theories of cell-multiplication will be used in the present paper:

- Exogeny
  - by partitioning
  - by vacuolation
  - from granules

- Endogeny
  - with migration from the protoplast
  - without migration

- Cell-division
  - by partitioning

with constriction of the cell-wall
with formation of entirely new cell-walls
in the absence of cell-walls (division of the naked protoplast).

This classification is intended to be as logical, precise, and self-explanatory as possible, but the meaning of its terms must be more fully explained below. These terms were not used by the originators of the several theories or by their adherents. As we shall see, some of the terms used by the early students of the subject are in fact inappropriate, and would confuse the account given here.

It may be remarked at the outset that while exogeny and endogeny are unreal, the various methods of division mentioned in the classification occur in nature.

The present paper deals with the history of the discovery of the methods of cell-multiplication down to the time of general acceptance of the views summarized in the phrase, *Omnis cellula e cellula.* In the next paper in the series it will be necessary to tell the story of the discoveries culminating in the generalization, *Omnis nucleus e nucleo.* The derivation of cell from cell and nucleus from nucleus will lead us back to the cell that originates a new individual. To complete the discussion of Proposition III it will therefore be necessary in the succeeding paper to show how it was discovered that the fertilized ovum is a cell formed by the fusion of two cells.

THE SUPPOSED ORIGIN OF CELLS BY EXOGENY

In Grew's little book, *The anatomy of vegetables begun* (1672), there is an interesting passage bearing on the subject of the origin of cells. It has already been mentioned in Part I of this series of papers (Baker, 1948) that Grew demonstrated the cellular nature of plant-embryos, and he must have realized that the adult plant contains an immensely greater number of cells (or 'Pores', as he often called them). He does not mention this subject specifically, but it seems to have been at the back of his mind when he wrote these words: 'In the *Piths* of many Plants, the greater Pores have some of them lesser ones within them, and some of them are divided with cross Membranes: And betwixt their several sides, have, I think, other smaller Pores visibly interjected' (p. 79). Thus Grew seems to have thought that new cells might originate in various ways. Visible interjection of new pores between the sides of old ones must presumably mean the origin of new cells by exogeny, but unfortunately he gives no details that would enable us to classify this supposed method of cell-multiplication more exactly. These words of Grew constitute the earliest reference to the problem of the multiplication of cells. I have already called attention to them elsewhere (1951, 1952*b*).

Exogeny by partitioning

The supposed origin of cells by exogenous partitioning is illustrated diagrammatically in fig. 1. A space between existing cells enlarges; partitions begin to appear in this space; they become more evident; new cells thus originate, and these enlarge.

The opinion that cells multiply by exogenous partitioning was put forward by Link (1807, p. 31). He later repeated his opinion that new cells originate in this way (1809–12, vol. 1, p. 7). He received little support, however, from subsequent writers, though Mirbel's *développement inter-utriculaire* and *super-utriculaire* (1835, p. 369) may perhaps fall into this category of theories. Mirbel's ideas were confused at the time by his firm belief that the whole of the 'membranous tissue' (that is, all the cell-walls) of a plant was perfectly continuous (see Baker, 1952*a*, p. 160), and the meaning of what he wrote on the subject of cell-multiplication is not clear.

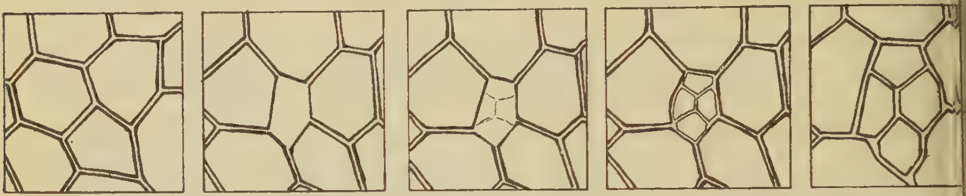


FIG. 1. Diagram of exogeny by partitioning. In this and in all the succeeding diagrams (figs. 2–6), the earliest stage is represented in the square on the left side, and the sequence of events is shown in the remaining squares from left to right across the figure.

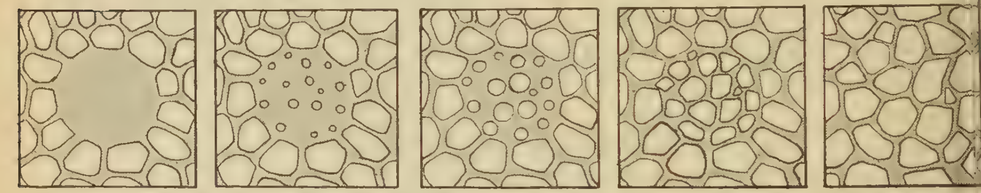


FIG. 2. Diagram of exogeny by vacuolation. The solid intercellular substance is shaded.

Exogeny by vacuolation

This is represented in fig. 2. Between the cells there is a homogeneous, solid or semi-solid substance. In places where there is much of this substance, minute vacuoles sometimes appear in it. These enlarge and transform themselves into new cells resembling the old.

Grew seems to have thought that cells might originate in some such way as this. He remarks (1682, p. 49) that when the sap penetrates into the seed, the liquid internal parts of the latter become coagulated into a solid; a process of 'Fermentation' transforms the coagulum 'into a *Congeries* of *Bladders*: For such is the *Parenchyma* of the whole *Seed*'.

It was Wolff (1759), however, who described exogeny by vacuolation most explicitly. His erroneous views on this subject formed the basis for his theory of epigenesis. He believed that the growing parts of plants were formed of a 'pure, homogeneous, glassy substance' (*pura æquabilis vitrea substantia*, p. 13);

another place he calls it a 'delicate, solid substance' (*substantia tenera olida*, p. 17). This material permitted the passage of nutritious fluids (p. 17). inute holes (*punctula*, p. 19), widely separated from one another, were formed it from blebs (*bullulae*, p. 17) of nutritious fluid; these holes swelled to ecome cells (*vesiculae*, p. 15; *cellulae*, p. 19). The glassy substance remained s the *interstitia* between them (p. 8). 'Leaves therefore grow for the most art by the interposition of new vesicles between the old, though partly indeed lso by the enlargement of the [existing] vesicles' (p. 14).

Wolff did not homologize the *globuli* constituting the blastoderm of the leveloping hen's egg with the *vesiculae* or *cellulae* of plants. On the contrary, e thought that the material composed of globules, despite its lack of homogeneity, was the counterpart of the glassy interstitial substance of plants; and

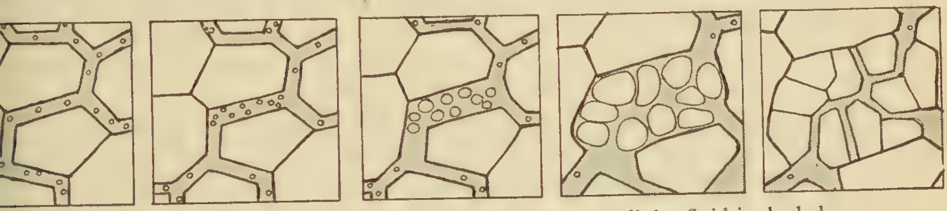


FIG. 3. Diagram of exogeny from granules. The intercellular fluid is shaded.

he thought that the cellular parts of the embryo (the *cellulosa animalis*)—that is, the viscera and vessels—were laid down epigenetically *in* this substance formed of globules (pp. 72, 75).

If one is to hold a balanced view of the history of the epigenesis-preformation controversy, it is necessary to grasp firmly what Wolff's views on the subject of cell-multiplication really were. The opinion, so commonly expressed, that Wolff was essentially right and Bonnet wrong, cannot be substantiated by a study of their writings. I have discussed this matter elsewhere (Baker, 1952*b*, pp. 183–6).

Exogeny from granules

This is represented in fig. 3. Small granules originate in the intercellular fluid; they expand, press upon one another, and become new cells.

It was from a study of the cotyledons in the germination of the seed that Sprengel (1802, pp. 89–90) derived his opinion that new plant cells originated from granules that subsequently enlarged. The granules in the cotyledons were in fact presumably starch-grains. It would appear that in his opinion the cell-forming granules sometimes originated inside and sometimes outside pre-existing cells, but unfortunately he is not explicit on this point. L. C. Treviranus adopted Sprengel's view (1806, pp. 2, 6–10, 14–16). He says that the intercellular spaces of plants contain a fluid that sometimes precipitates fine granules; these grow into *Blasen* or cells. For him, indeed, the purpose of the intercellular fluid (*Saft*) was to produce new cells: it carried the granules wherever new cellular tissue was to be formed. It did not surprise him that the granules that were to become cells were sometimes seen within cells because he believed that there was free communication between the intercellular fluid and the cavities of the cells, through apertures in the cell-walls

Rudolphi (1807, p. 35) considered that the intercellular fluid could form new *Bläschen*. He supported Sprengel in general, but gave no particulars.

After a long period of eclipse, the theory of exogeny from granules was reintroduced in the eighteen-thirties and became famous through its promulgation, in a modified form, by Schwann. Valentin (1835, p. 194) first gave a curious account of the origin of the pigment of the chorioid coat of the eye of birds and mammals. Colourless, transparent bodies that he called by the misleading names of *Pigmentkörperchen* and *Pigmentbläschen* appeared first, and the actual globules of pigment subsequently developed in aggregations round each of them. Four years later (1839, p. 133), Valentin announced that the *Pigmentbläschen* were in fact nuclei, and that cells containing pigment were formed round the nuclei after the latter had appeared. This led to a dispute with Schwann (1839, p. 264) about priority.

Schwann, as is well known, considered that new cells originate in a structureless substance which he called the *Cytoblastema* (1839, p. 45) or *Cytoblastem* (p. 112 and elsewhere). This substance, he supposed, sometimes existed within pre-existing cells, but in animals it was usually extracellular (pp. 203–4). Its consistency differed in different cases. It was often fluid, but might also be solid: the matrix of cartilage was an example of it. Schwann's general scheme of cell-formation was as follows (1839, pp. 207–12). The first object to appear in the previously homogenous *Cytoblastem* was the nucleolus. A clump of granules next appeared round this; these then resolved themselves into a pellucid nucleus with a clear boundary, which sometimes took the form of a distinct membrane. The nucleus grew. When it had reached a certain size, a substance derived from the *Cytoblastem* was deposited on it in the form of a layer. Either the whole of this layer, or the outer part of it only, was the future cell-wall (*Membran*). The nucleus adhered to the cell-wall in one place, but elsewhere a fluid appeared between the two and separated them; this fluid, the *Zelleninhalt*, increased in volume. The typical nucleated cell was thus produced. The nucleus in most cases was eventually absorbed and disappeared.

In forming his opinions about the origin of cells, Schwann was undoubtedly much influenced by Schleiden, though he placed the *Cytoblastem* of animal cells, as a general rule, outside pre-existing cells, while Schleiden regarded cell-formation in plants as endogenous (see below, p. 416).

The exogenous origin of cells in a *Cytoblasteme*, as he called it, was reiterated by Vogt in his book on the development of the obstetric toad, *Alytes* (1842). Vogt distinguished between *Cytoblasteme primäre*, or intercellular material that had never formed part of a cell, and *Cytoblasteme secondäre*, formed of material that had previously composed cells and had subsequently become structureless (p. 125). He held that cell-formation started in *Alytes* when cleavage was finished (pp. 9–10, 25). In the *Cytoblasteme* (whether primary or secondary) a nucleus originated, and round this a cell (pp. 117–19); sometimes he cell originated first (pp. 119–20). Vogt does not describe the details of the rocess, but the nucleolus was not the first object to appear (p. 118).

It is a remarkable fact that so late as 1849, Virchow instituted a comparison etween the origin of a crystal from its mother-liquor, and a cell from the *Blastem*. 'Both the mother-liquor and the *Blastem* are amorphous substances, from which bodies of definite shape arise by aggregation of atoms' (1849, pp. 8–9). The difference lay in the substance of the crystal being already present in the liquor, while chemical change was necessary for the differentiation of the cell.

THE SUPPOSED ORIGIN OF CELLS BY ENDOGENY

Endogeny with migration from the protoplast

Fig. 4 represents the origin of a new cell in a filamentous alga by this hypothetical method. In such a form as this there are no intercellular spaces, and exogeny is therefore scarcely possible. The cells contain granules. These have the property of being able to migrate through the cell-wall and grow into new cells; in these, new granules appear endogenously, which are capable of repeating the process.

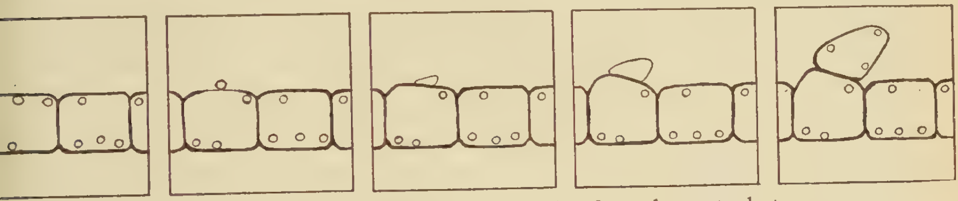


FIG. 4. Diagram of endogeny with migration from the protoplast.

L. C. Treviranus supposed that this method of cell-multiplication occurred in certain algae. He considered that the new tubes (*Schläuchen*) of the water-net, *Hydrodictyon*, arose from granules that were present on (*an*) the walls of the old tubes (1806, p. 3). He did not give particulars of the original positions of the granules, but what he saw were probably the pyrenoids, which are very evident in this plant. He derived the new cells of filamentous algae from the chloroplasts originally situated within pre-existing cells (1811, p. 6).

Kieser's writings (1814, pp. 105, 219) on cell-multiplication are not very explicit. He derived new cells from the small globules that are found in the *sève* contained in the intercellular spaces. He appears to have supposed that these globules originated within cells. Turpin (1829, p. 181) considered that Kieser's globules must have originated within cells; he allowed that they might perhaps develop into new cells in the intercellular spaces, in certain cases. As we shall see, however (p. 415), Turpin thought that new cells usually developed within pre-existing cells, and that no migration took place.

Endogeny without migration

According to this theory, which was supported by a formidable literature, small granules originate within a pre-existing cell (fig. 5); these granules enlarge at the expense of the contents of the pre-existing cell, until they touch one another and take on the usual characters of cells. The cell-wall of the mother-cell eventually disappears.

Grew's remark, quoted above (p. 409), that 'the greater Pores have some of them lesser ones within them', suggests that he may have envisaged this as a possible method of cell-multiplication. The theory, however, was first put forward in concrete form by Sprengel, in his account of the germination of the bean-seed (1802). He gives an illustration of cells of the cotyledon, with small granules or vesicles within them (his plate I, fig. 2), and he remarks, 'The small vesicles that still float in the fluid of the cell seem to have the character of future cells, and perhaps will become transformed into them subsequently' (p. 90). The granules or vesicles were actually starch-grains, the nature of which was not in the least understood at the time. In his book published in 1811, L. C. Treviranus also derived new cells from the granules contained inside the cells of the cotyledons of beans and peas (p. 4); in this later work he does not dogmatize as to the place of origin of these granules. Kieser (1814, p. 219) seems to have thought that the small globules that originate within cells and are the primordia of new cells, sometimes undergo their transformation without first passing out into the intercellular *sève*.

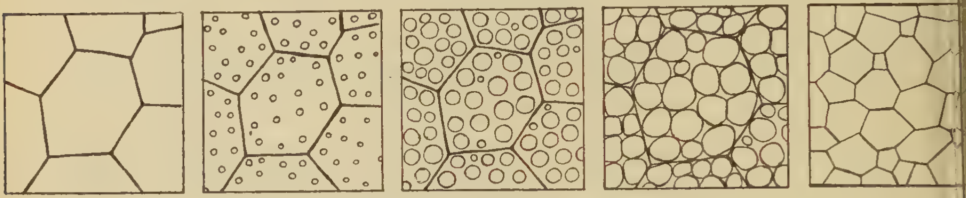


FIG. 5. Diagram of endogeny without migration.

The theory of endogeny without migration flourished in the eighteen-twenties through the labours of Raspail and Turpin. From his studies of the germination of cereals, Raspail concluded that new cells arose from starch-grains, which enlarged within the cell that produced them until they touched one another, the mother-cell eventually bursting (1825, pp. 412–13). It is strange that the very man who discovered, by the iodine test, that these granules contain starch, should have been so misled about their fate. He elaborated his views in further communications. He imagined that each starch-grain contained within itself one or more globules, and that there were other exceedingly minute ones inside these (1827, p. 212, and his plate 2, fig. 22); this kind of *emboîtement* at the cellular level provided for repeated acts of cell-multiplication. He derived a whole leaf from a single cell, inside which two new cells arose endogenously and enlarged so as to fill all the space except what would become the midrib; globules arose within these two cells and enlarged to fill all the space except what would become the veins; and so on, till the final cellular structure of the mature leaf was achieved (1827, pp. 254–5, and his plate 4, fig. 4). He applied the same idea to the stems of plants

p. 269) and to the tissues of animals (p. 304). He repeated his opinions on the ndogenous origin of new cells, in his book on biochemistry (1833, pp. 85–86).

Turpin had completed his first paper on cell-multiplication by endogeny vhen he received Raspail's original communication on the subject (Turpin, 827*a*, pp. 47–48). One may summarize his views, which he put forward at great length, by saying that he derived new cells from chromatophores and rom colourless bodies which he believed to be of the same nature. He regarded *Pleurococcus naegelii*, so abundantly found on damp walls, as a solitary chromatophore. This plant, which he called a *globuline*, was for him a typical example of the most primitive organisms. The globuline contains within it a large number of smaller globulines, destined to reproduce the little organism (p. 25). Most plants, however, consist of colourless cells, which *contain* globulines; the latter are commonly green, but the starch-grains of the potato, for instance, are examples of white globulines (p. 42). Each globuline has the latent capacity to swell within its parent cell to form a new cell, losing its colour (if any) in the process (p. 41).

Turpin now investigated particular plants in the light of his ideas on cell-multiplication (1827*b*, 1828*a*). He derived new branches of *Enteromorpha* (which he called *Ulva*) from globulines situated within the cells. It is impossible to be certain of the real nature of these particular globulines; possibly they were zoospores. He gives a figure showing three branches of the plant that have originated from globulines all situated in a single cell (1828*a*; his plate 11, fig. 3).

In an extraordinary and tantalizing paper (1828*b*), Turpin mentions the great number of cases, both in simple microscopical plants and in the reproductive parts of higher forms, in which cells occur in aggregations of 2, 4, 8, or 16. He cites the pollen mother-cells of *Cobæa* (see below, p. 432). One would have thought that the idea of repeated binary fission would have forced itself on his imagination; but no, he thinks there is some unexplained tendency towards the germination of globulines in these particular numbers.

Turpin finally summarized his views at considerable length (1829), without adding anything of importance.

The ideas of Raspail were carried over into the embryology of animals by de Quatrefages (1834), in his study of the development of the pulmonate gastropods of fresh water. He thought that the early blastomeres or *globules* contained small similar bodies which grew and distended them, and that the process was repeated until a mass of cells had been produced, which took the form of the little mollusc (p. 115). Dumortier (1837), a student of the embryology of the same group of animals, appears to have held somewhat similar views. Misunderstanding the cleavage stages, he regarded the early embryo of *Limnaea* as being merely lobed and later facetted on the surface. He thought that cells appeared for the first time about a week after these stages. The cells that then appeared in the interior of the embryo he called *cellules primitives*. Inside each of these there arose eight or more *cellules secondaires*, indicated at first by certain *striatures obscures*. These secondary cells appear to have enlarged at the expense of material contained within the parent cell, until they filled it. (If, however, the striations divided the whole of the primary cell into secondary cells, the method should strictly be described as cell-division by partitioning (see below, p. 419).) The secondary cells subsequently enlarged until the primary cell burst, and only remnants of it were left. Only certain parts of the animal were formed of cells: the head was not (Dumortier, 1837, pp. 137 and 143–50, and his plate 4, fig. 16*a*).

The contributions of Schleiden to this subject must be considered in some detail, because they had a strong influence on contemporary opinion. He remarks (1838, p. 161) that new cells must either be formed outside the existing mass of tissue, or in its interior; if the latter, either in the intercellular spaces, or in the cells themselves; there is no fourth possibility (*quartum non datur*). The development of the plant occurs solely by the formation of cells within pre-existing cells and their subsequent expansion (pp. 163–5). He studied the development of new cells especially in the endosperm and pollen-tube. It may be remarked that he could scarcely have chosen an object of study more likely to lead him astray than the endosperm; for the development of a syncytium, with subsequent division into cells, does in fact bear some resemblance to the supposed process of endogeny without migration. The pollen-tube was almost as likely to lead to misinterpretation.

Schleiden gives a general account of the origin of new cells in these two situations. He describes the embryo-sack, in which the cells of the endosperm are to arise by endogeny, as a *Zelle* (p. 144). The first sign of impending cell-formation in the cytoplasm or *Gummi* of this cell, or of the pollen-tube, is the appearance of small mucus-granules. The nucleoli, larger and more sharply defined than the more numerous mucus-granules, are the next objects to appear. Schleiden calls them *Kernchen* (p. 145) or *Kerne der Cytoblasten* (p. 174); the descriptions and figures leave no doubt as to the correct interpretation of these names. It is unfortunate that in their translations of Schleiden's paper into English, both Francis (see Schleiden, 1841, p. 287) find Smith (see Schleiden, 1847, p. 238), overlooked what the original author said about the role of the nucleolus, apparently because they misread *Kernchen* as *Körnchen*; as a result, Schleiden's views have not till now been adequately represented to English readers. According to Schleiden himself (1838, p. 145), the nucleolus is the body round which the nucleus is formed, by the deposition in its immediate vicinity of a granular coagulum. (It is not clear whether the mucus-granules participate in this coagulum.) Schleiden called the nucleus the *Cytoblastus* (p. 139) or *Cytoblast* because he thought that its function was to produce the cell. According to his account (pp. 145–6) it grows larger, and a little blister, the rudiment of the future cell, appears on its surface. The contents of the blister are transparent. The appearance is rather like that of a watch-glass on a watch. The blister enlarges so as eventually to enclose the nucleus, except on one side. Its wall becomes stiffened into a jelly. When this process is complete, the blister has become a cell, the nucleus remaining enclosed in one place in its wall. The cell grows and assumes a regular shape s a result of the pressure of the other new cells surrounding it. The nucleus enerally disappears after the cell has assumed its final form.

In his first paper on the cell-theory, Schwann (1838*a*) maintained that chleiden's statements about the way in which cells multiply applied also to nimals. He claimed to have found small cells within larger ones in the otochordal tissue and cartilage of the larvae of the spade-footed 'toad', *elobates*. In his book published the next year, he allowed that in animals new ells sometimes develop inside pre-existing ones, but he thought an exogenous rigin much more usual (1839, pp. 45, 200, 203–4; see above, p. 412).

In his study of the earliest stages in the development of the rabbit, Barry oncluded that two or more 'vesicles' (cells) originate within each pre-existing one (1839, p. 363). This is surprising, because he compares the early embryo with that of the frog; and he already knew, from the studies of Prévost and Dumas (1824), that in the latter animal the number of blastomeres increases by binary fission. Barry did not state clearly how he thought that cells multiply, although he mentioned the subject in several papers (1841, *a*, *b*, and *c*), which are unsatisfactory in more than one respect. He seems to have thought that the nucleus divides or fragments, and that each part of it grows to become a new cell.

Reichert considered that the blastomeres of amphibian eggs were formed endogenously within the uncloven egg, and smaller blastomeres endogenously within these, and so on: cleavage was merely the separation of blastomeres that were already present (1840, p. 7; 1841, p. 540).

Henle described what he thought to be a new cell arising endogenously round a nucleolus, within an existing (? human) cartilage-cell (1841, pp. 153–4 and his plate V, fig. 6). It is just possible that he was in fact observing a stage in cell-division.

Vogt considered that new cells sometimes arose within pre-existing cells, even on occasions within their nuclei (1842, pp. 126–7); but he thought that in animals exogeny was the more usual process (see above, p. 412). He regarded the nucleolus of the egg as a cell embedded in another cell, the nucleus, itself embedded in a third, the yolk (1842, p. 18).

A curious misapprehension prevented Kölliker from being among the first to understand the true nature of blastomeres. In his researches on the development of nematodes (1843), he got the fixed idea that the cells of the later embryo originate from what were really the nuclei of the earlier stages. These nuclei he called *Embryonalzellen*, to emphasize his opinion of their nature, and their nucleoli he regarded as nuclei (*Kerne*) (pp. 101–2). In some cases, in his view, there was no cleavage, but only a multiplication of the *Embryonalzellen*. Each of these produced two new small ones endogenously within it, and dissolved to set them free; the same process then happened repeatedly until all the numerous cells of the later embryo had been produced (p. 79). In other cases (e.g. in what he called *Ascaris nigrovenosa* (presumably another name for what is now called *Rhabdias bufonis*)), he described cleavage clearly enough, and gave excellent figures of it (his plate VI, figs. 21–23); but he totally misunderstood it. He knew that the blastomeres multiplied by division, but he was evidently not much interested in them, for he regarded them as mere spherical conglomerates of yolk-granules (pp. 105–6) round the all-important *Embryonalzellen*, which were going to multiply and produce the definitive cells.

In his general account of the multiplication of cells, published the next year in his book on the development of cephalopods (1844, pp. 141–57), he called the cells of adult animals *secondäre Zellen*, while nuclei were for him *primäre Zellen*. The nucleolus was the *Kern* of the primary cell. A blastomere was not a cell but an *Umhüllungskugel*. His views may be translated into modern terms as follows. In the early embryo, the nucleus of each blastomere ordinarily gives rise to two nuclei, by aggregation of material round the nucleoli. A substance which may be either granular or homogenous aggregates round each nucleus; the two aggregates separate from one another by a process involving the division of the original blastomere into two. This process continues until at last definitive cells are produced by the formation of cell-walls; these appear either round blastomeres, or else round nuclei. Kölliker also thought that a definitive cell might produce daughter-cells endogenously, apparently in the cytoplasm, and then degenerate to set them free. He also thought that new cells might arise within a mass of material formed by the fusion of cells.

Kölliker denied specifically that cells multiply by division. Having at last (1845) adopted a more acceptable nomenclature, he remarked without equivocation, 'Nothing whatever is known of a division of animal cells. Nuclei and cells multiply by endogenous procreation, nucleoli (*Kernchen*) by division' (p. 82). These are strange words from one who had observed cleavage so accurately.

In his old age, Kölliker claimed that in his book on the development of cephalopods (1844), he had made it very probable that all cells are the direct descendants of the blastomeres (Koelliker, 1899, p. 198). The truth is that in the book to which he refers he gave a confused and erroneous account of the way in which cells multiply, while the actual facts, as we shall see (pp. 430–1), had already been revealed by Bergmann in 1841–2.

J. Goodsir (1845, p. 2) regarded the nucleus as the source of successive broods of new cells, which grew within the mother-cell. It is not clear, however, whether he thought the new cells remained within the mother-cell or escaped from it. Goodsir attributed the discovery of the method by which cells multiply to Barry.

Beale (1865, pp. 241–2) appears to have been the last exponent of endogeny, though his remarks on the subject of the multiplication of cells are difficult to understand. He derived new 'elementary parts' (as he called cells) from minute particles, present (it would seem) within pre-existing cells. These particles enlarged, and meanwhile other similar particles might arise within them and also grow, and so on. This would be a clear example of endogeny without migration, but apparently the whole mass might divide and subdivide. When Beale reached these rather elusive conclusions, however, others had already discovered how cells actually multiply.

THE DISCOVERY OF CELL-DIVISION

Preliminary remarks

About the middle of the nineteenth century there occurred a profound change in the beliefs of biologists about the way in which cells multiply. This change cannot be more dramatically recorded than in two extracts from the writings of Virchow. The first is from an original paper published in 1849. The second is the corresponding passage in a book of his collected papers, published seven years later. In the following translation an attempt is made to reproduce the style of Virchow's early writings, which are reminiscent of Oken's *Naturphilosophie.*

'The cell, as the simplest form of life-manifestation that nevertheless fully represents the idea of life, is the *organic unity*, the indivisible living One' (1849, p. 8).

'The cell, as the simplest form of life-manifestation that nevertheless fully represents the idea of life, is the *organic unity*, the divisible living One' (1856, p. 22).

In a note added to the second publication (p. 27), Virchow tries to persuade us that when he wrote *untheilbare* in 1849, he used the term in a philosophical rather than a scientific sense. It is difficult to accept this. There had, in fact, been a revolution in thought. It is the purpose of the rest of this paper to tell the story of this revolution and of the events that led up to it.

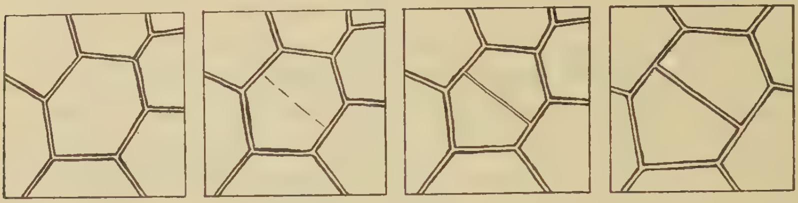
The early cytologists drew sharp distinctions between various methods of cell-division that seem to us to be very similar in all essential points. So sharp did these distinctions appear to them, however, that they would even describe cell-division while denying that cells ever divided. The difficulty is really verbal. They concentrated their attention on the cell-*wall*: this was for them the cell. If the wall did not divide, the cell did not divide, whatever might happen to its 'contents'.

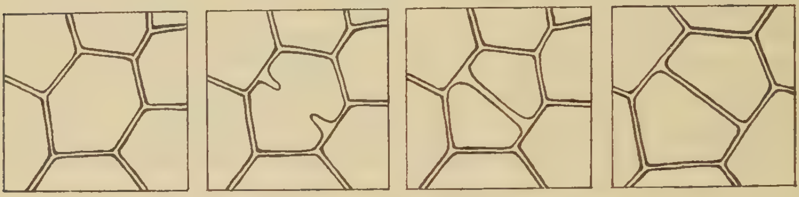
Four methods of cell-division are illustrated diagrammatically in fig. 6.

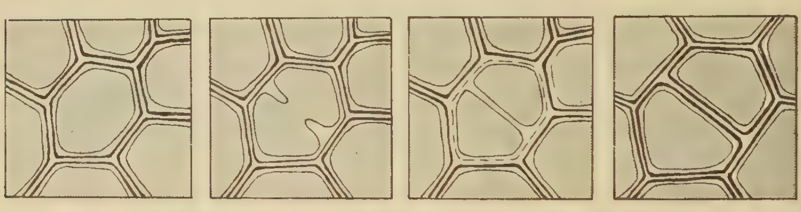
In *cell-division by partitioning*, a thin membrane appears across the middle of a cell. It thickens and is seen to be a double partition, continuous with the pre-existing cell-walls. A single cell has become two cells, each of half the original volume. These grow.

In *cell-division with constriction of the cell-wall*, the latter bends inwards on all sides near the middle of the cell; a continuation of this process results in the division of the whole cell, including its wall. The two new cells grow. This was regarded as genuine cell-division by the early cytologists. It was called *Theilung durch Abschnürung*.

In *cell-division with formation of entirely new cell-walls*, the protoplasm divides into two or more parts inside the wall of the pre-existing cell. Each of these parts grows and acquires a complete new wall of its own, while the original wall disintegrates. This was the *Zellenbildung um Inhaltsportionen* of the early German cytologists. This name was given because the 'cells' (actually the cell-walls) were formed afresh round portions of the cell 'contents' (protoplasm). In some cases the protoplasm divided up into numerous bodies that did not touch one another nor the wall of the mother-cell, and a new cell-wall was formed *separately* round each of these bodies. Since these new cell-walls were 'free' from each other, the name *freie Zellbildung* was used.


Cell-division by partitioning


Cell-division with constriction of the cell-wall


Cell-division with formation of entirely new cell-walls

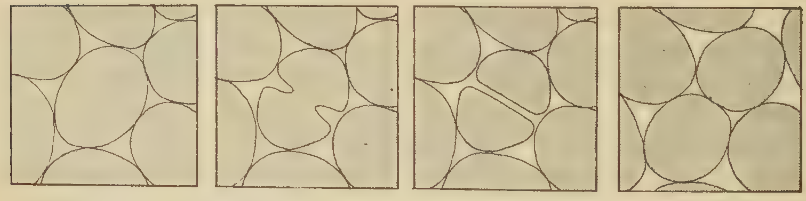

Cell-division in the absence of cell-walls (division of the naked protoplast)

FIG. 6. Diagrams of methods of cell-division. In the two lower diagrams the protoplasts are shaded.

Cell-division with the formation of entirely new cell-walls shows a certain legree of resemblance to endogeny without migration. In endogeny, however, he new cells were supposed to originate as minute bodies that grew within the ytoplasm of the mother-cell, while in fact, of course, cells are first formed by he process of division, and growth is subsequent to this.

The endosperm is the classical site for the study of cell-division of this third type.

In *cell-division in the absence of cell-walls* (*division of the naked protoplast*) a furrow appears round the middle of a protoplast that has no cell-wall, and deepens until division is complete; the two resulting protoplasts then grow. This method of cell-division could not be envisaged until it was discovered that the cell-wall was not a necessary attribute of the cell. The history of that discovery was related in Part III of this series of papers (Baker, 1952).

Various different lines of research led up to the discovery that cells multiply by division into two or more parts. The chief of these were studies of protists, filamentous algae, and cleaving eggs. It would be possible to relate the story by concentrating first on examples of cell-division by partitioning, then on examples of division with constriction of the cell-wall, and so on; but the differences between the four methods result from such an unwarrantable overstressing of the cell-wall, that an unsatisfactory history would result. A far more logical arrangement will be to take each line of research separately. We shall begin with the results of researches on protists, for it was among these organisms that the process of cell-division was first witnessed.

The multiplication of protists

Leeuwenhoek saw ciliates coupled in pairs on several occasions (1681, p. 57; 1694, p. 198; 1697, p. 36; 1704, p. 1311). He interpreted the process in every case as one of copulation. Once he saw them actually come together in pairs *in conspectu meo* (1697, p. 36), and he must therefore have witnessed a stage in conjugation; but he does not give sufficiently accurate descriptions to make it certain whether he witnessed a stage in division on one or more of the other occasions. It is possible that he did (see especially 1694, p. 198). He himself, however, had no idea that ciliates multiply by division. He thought, on the contrary, that they reproduced by minute round particles (1697, p. 36), which in fact were presumably food-vacuoles (see also 1681, p. 56).

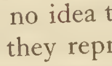


Fig. 7. The earliest illustration of a protist in division. (Anon., 1704, plate opposite p. 1329, fig. G (*c*).)

The first figure of a ciliate in division was given by an anonymous contributor to the Philosophical Transactions of the Royal Society (Anon., 1704; see fig. 7 in the present paper). The author himself did not regard this as a stage in multiplication by division. On the contrary, he compared the appearance with that of flies in copulation (pp. 1368–9). From what he says, it is quite possible that he saw ciliates in stages of both conjugation and division.

Joblot seems also to have seen a stage in the division of an unidentifiable ciliate (1718, plate 2, fig. 5). Like the anonymous writer, he regarded it as representing two individuals 'accouplées' (1718, part 2, p. 14), and indeed he appears to have seen stages in conjugation (his plate 2, fig. 1, and plate 3, fig. 9).

The first person to witness the process of multiplication of a protist by division was Trembley. He saw it in 1744 in the colonial vorticellid *Epistylis anastatica* and in *Stentor* (Trembley, 1746), and later in *Carchesium* and *Zoothamnium* (1748). I have described these discoveries in detail elsewhere (Baker, 1952*b*, pp. 103–12). It must suffice to say that Trembley's studies of these organisms, carried out with extraordinary care and accuracy, established for the first time the method of multiplication of Protozoa and provided a firm basis for disbelief in their spontaneous generation. The ciliates, however, can scarcely be considered as cells, in the sense in which that word is being used in this series of papers, on account of the highly polyploid nature of the macronucleus (see Baker, 1948*b*); and although this early work of Trembley's paved the way for the understanding of cell-division, yet it was not an investigation of cell-division itself. We shall therefore not pursue the subject of the reproduction of ciliates here, beyond remarking that Spallanzani, who corresponded with Trembley, also saw stages in their multiplication by division (Spallanzani, 1776, part 1, pp. 160 and 174–5, and his plate I, fig. VII, and plate II, figs. XIII and XIV).

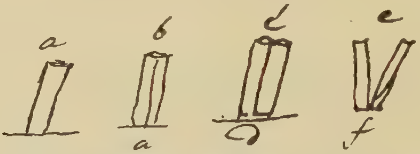


FIG. 8. The earliest illustration of cell-division. Trembley's sketch of the diatom *Synedra* dividing into two. (Trembley, 1766, folio 330.)

Meanwhile Trembley himself had seen actual cell-division in the sessile, rod-shaped, fresh-water diatom, *Synedra*. I have described this discovery in detail elsewhere (Baker, 1951; 1952*b*, pp. 155–8). Trembley's sketch of the process is here reproduced in fig. 8. He noticed that a line appeared along the length of the organism, and became more conspicuous; then the whole object appeared to become a little wider, and the line was seen to be a groove; the parts on each side of the groove rounded themselves off from one another, and the previously single body was then seen to be double; finally the two halves of the originally single body diverged from one another at the unattached end. Trembley described this process first in a letter to a friend (1766, folio 330), and then, much later, in a book intended for the education of children (1775, vol. 1, pp. 293–7); in the interval another friend, Bonnet, had named the organism the *Tubiforme* and published the main facts of Trembley's discovery in his *Palingénésie philosophique* in 1769 (vol. 2, pp. 99–102). Trembley also observed cell-division in a stalked diatom, named by Bonnet the *Navette*;

his was almost certainly *Cymbella* (see Bonnet, 1769, vol. 2, pp. 104–5; Trembley, 1775, vol. 1, p. 297). Neither Trembley nor anyone else in his ime realized that such organisms as *Synedra* and the component individuals of a *Cymbella* colony were cells.

Certain of Gleichen's figures suggest that he may have seen stages in the multiplication of the non-ciliate Protozoa of infusions, but the drawings are not clear enough to establish this (1778; see, e.g., his plate XVII, figs. C III and D III).

O. F. Müller described a dividing specimen of the desmid *Closterium*, which he called *Vibrio lunula* (1786, p. 57). His illustration is reproduced here in fig. 9. He also showed stages in the longitudinal division of a little organism found in stale sea-water, which may possibly have been a flagellate (his plate VIII, figs. 4–6).

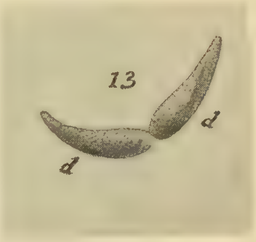


Fig. 9. O. F. Müller's figure of *Closterium* dividing into two. (Müller, 1786, plate VII, fig. 13).

The first person to describe the division of a protist with full realization that the process was one of cell-division, was Morren (1830). He describes *Crucigenia quadrata* as being ordinarily composed of *cellules* united in fours (see fig. 10). He describes the division of a single cell to form four, and of the four to form 16 (pp. 415–22). Later Morren saw stages in the multiplication of *Closterium*. He describes the extension inwards of a circular plate that makes a partition across the organism, which becomes jointed in this region; *déhiscence* then takes place (1836, p. 274).

Meanwhile Ehrenberg had started his celebrated researches. He described and figured an *Actinophrys* in the process of division (1830, p. 96); his illustration, here reproduced in fig. 11, A, suggests that the specimen was genuinely *Actinophrys*, not *Actinosphaerium*. Later (1832, p. 178) he saw a series of stages in the multiplication of *Euglena acus* by longitudinal fission (see fig. 11, B in the present paper). In his book on *Die Infusionsthierchen* (1838) he described and figured a number of examples of the multiplication of flagellates by division; for example, *Polytoma* (p. 25 and his plate I, fig. XXXII), *Pandorina* (p. 54 and plate II, fig. XXXIII), and *Glenodinium* (p. 257 and plate XXII, fig. XXII).

Nägeli described and figured the multiplication of certain diatoms by division (1844, plate I, figs. 1–6).

Cell-division in simple filamentous algae

The simplicity and immobility of most filamentous algae made them particularly suitable objects for the discovery of the way in which cells multiply.

In his careful researches on fresh-water algae, Vaucher at last succeeded in observing the germination of a zygote of *Spirogyra* (which he called *conferva jugalis*). He describes (1803, p. 47) how the cell-wall of the zygote (*grain*) opens at one end; a sack extends from it and begins to elongate into a tube.

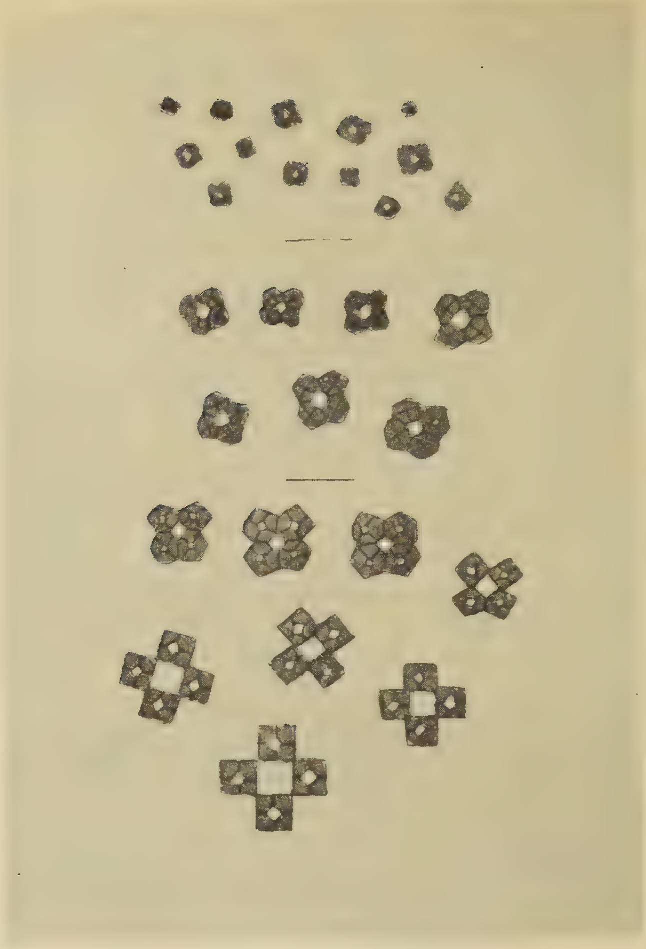


FIG. 10. Morren's figures of cell-division in *Crucigenia*. (Morren, 1830, plate 15, figs. 3–5.)

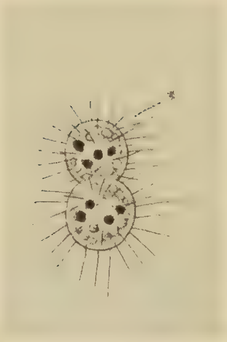


FIG. 11, A

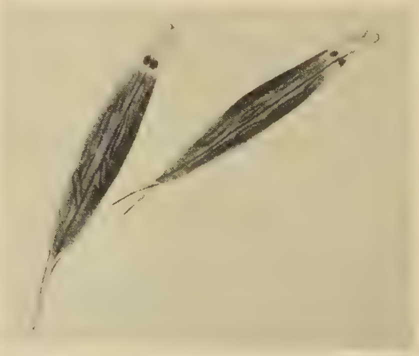


FIG. 11, B

FIG. 11. Ehrenberg's figures of unicellular organisms in division. A, *Actinophrys sol*. (Ehrenberg, 1830, plate II, fig. IV (6).) B, *Euglena acus*. (Ehrenberg, 1832, plate I, fig. III (*b*, *c*). The plate (not the text) is dated 1831.)

He notes how the partitions (*cloisons*) between the cells (*loges*) appear: first one, then two, then many, until finally the tube resembles the plant that gave birth to it. His illustration is reproduced here (fig. 12). In his plate X, fig. 3, Vaucher shows stages in the germination of another alga; the number of partitions is seen to increase. The book contains nothing about the *binary* fission of the cells of any alga.

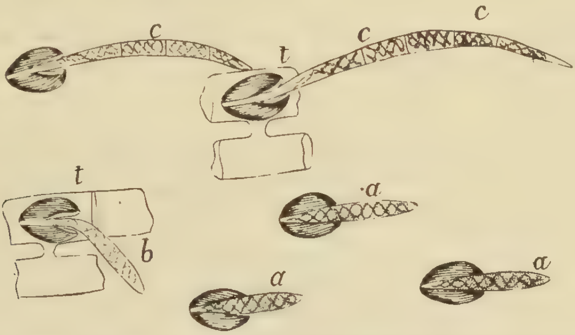


FIG. 12. Vaucher's figure showing new partitions between cells in a young *Spirogyra*. (Vaucher, 1803, plate IV, fig. 5.)

We have seen (p. 415) that Dumortier (1837) was mistaken about the way in which the cells of the embryo of *Limnaea* multiply. Five years previously, however, he had made an important contribution to the study of cell-multiplication in filamentous algae. He describes carefully (1832, pp. 226–7) how an extension inwards of the internal part of the cell-wall 'tends to divide the *cellule* into two parts'. He discusses whether the dividing wall or *cloison* is from the start double. He does not decide the question, but he says that in later stages it is certainly double in the conjugate filamentous algae. He supposed that cell-division was restricted to the cell at the extremity of a filament (see fig. 13 in the present paper). He remarks (p. 228) that new cells cannot originate from globules floating in the intercellular spaces, because some plants, such as the ones he was studying, have no such spaces.

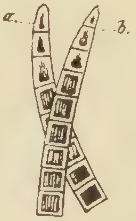


FIG. 13. Dumortier's figure of cell-division in *Conferva aurea*. '*a*, terminal cell that elongates more than the lower ones; *b*, the same divided into two parts by the formation of a median partition.' (Dumortier, 1832, plate X, fig. 15.)

Mohl's celebrated paper *On the multiplication of plant-cells by division* (1837) was first made public in the form of an inaugural lecture on his appointment as Professor of Botany at Tübingen in 1835. Like Dumortier, he studied filamentous algae (see fig. 14). His work on these organisms marks a turning-point in the history of the study of cell-multiplication, but he himself wrote with charming diffidence. 'Furthermore', he remarks, 'many appearances that I have observed in the various species of *Zygonema* [actually *Spirogyra*] make it seem to me more than likely that in

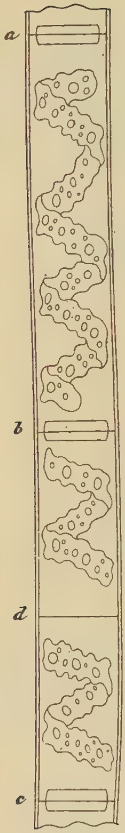


FIG. 14. Mohl's figure of cell-division in *Spirogyra*. The cell wall between the two newly formed cells is at *d*. (Mohl, 1837, plate I, fig. 8.)

these plants also the individual cells possess the capacity to divide themselves in the middle by a partition-wall formed subsequently. . . . The observations cited above will suffice to prove that the increase of cells by division is not an altogether rare phenomenon among the Confervae' (pp. 29, 30).

Meyen (1838, p. 345) described the multiplication of the cells of certain filamentous algae by the process called in the present paper 'cell-division with constriction of the cell-wall'. He referred to this process, and also to cell-division by partitioning, by the name of *Theilung*.

Cleavage, and the recognition of blastomeres as cells

The study of the cleavage of the eggs of animals played an important part in convincing biologists that cells multiply by division. It was fairly easy to show that blastomeres did so; the difficulty was to discover that they were cells.

Swammerdam is thought by some to have seen a 2-cell stage in the cleavage of the frog's egg. One of his illustrations is reproduced here (fig. 15). He had placed the egg in a special fluid intended to dissolve the jelly, and this had so distorted it that one can neither affirm nor deny that he saw a stage in cleavage. He wrote, 'Next I observed the whole of the little frog divided, as it were, into two parts by a very obvious furrow or fold' (1737–8, vol. 2, p. 813). He never saw cleavage occurring in a living egg, nor did he see the 4-cell or later stages of the process. His observations on the embryology of frogs were perhaps made while he was studying these animals at Leiden in 1661–3. Dr. A. Schierbeck, however, who has made a careful study of the life of Swammerdam, thinks it probable that these observations were made in 1665. They were first published in the *Biblia naturae* long after his death.

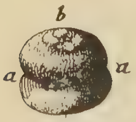


FIG. 15. Swammerdam's figure of a frog's egg, possibly at the 2-cell stage. (Swammerdam, 1737–8, vol. 2, plate XLVIII, fig. v.)

It was stated by Bischoff (1842*a*, p. 46) that de Graaf (1672) saw a 2-blastomere stage in the rabbit. This is not true. De Graaf examined the Fallopian tubes and uteri of rabbits at various intervals after coition. He neither describes nor illustrates any stage in cleavage. From the third day onwards for several days he saw what must actually have been blastocysts (and perhaps late morulae), gradually increasing in size (pp. 313–14, and his plate XXVI, figs. 1–5). He saw no indication of the constituent cells.

Rösel von Rosenhof (1758) studied the development of several species of Anura. It is possible that he saw the 2-cell stage in the tree-frog, *Hyla arborea* (see his plate X, fig. 5, and p. 43). He also gives figures suggesting that he saw the first cleavage-furrow in *Rana temporaria* (plate II, figs. 9 and 10), but the accompanying text (p. 7) shows that the embryos were too old for this to be possible, and the drawings presumably represent neurulae.

Roffredi (1775) saw cleavage-stages in the free-living nematode *Rhabditis*. He figured the nuclei, but evidently did not understand what he was observing, for he did not notice the boundaries of the blastomeres (see Baker, 1949).

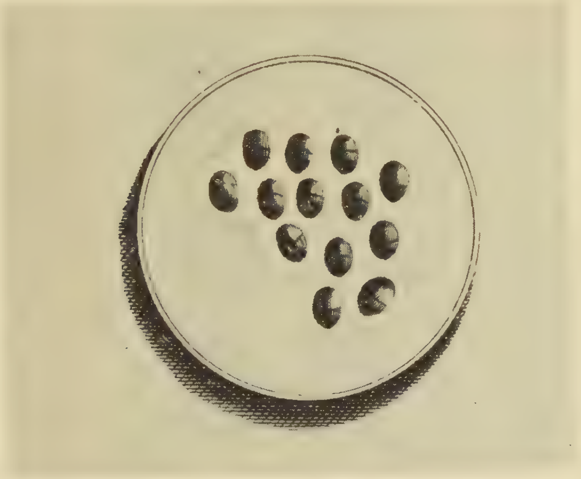


FIG. 16. Spallanzani's figure of the 4-cell stage in the development of the toad. (Spallanzani, 1780, plate II, fig. XI.)

It seems almost certain that Spallanzani (1780) saw the 4-cell stage in the toad (*Bufo*). His illustration is reproduced here in fig. 16. He calls the furrows *solchetti* (vol. 2, p. 25), and compares the appearance to that of the cupule of a chestnut when it has begun to split into its four lobes. He also gives what might be thought on casual reading to be a description of a 2-cell stage in the green frog (vol. 2, p. 13), but the *solco* was here actually the neural groove, not a cleavage-furrow. Spallanzani did not understand the process of cleavage. Indeed, he was so prejudiced by his belief in the actual pre-existence of later stages in the unfertilized egg, that he could not give any adequate account of the occurrences involved in development.

The process of cleavage, as it occurs in the living embryo, was first described by Prévost and Dumas in 1824. Their observations were made upon the eggs of what they called the *Grenouille commune*. This appears in fact to have been *Rana esculenta*, in which species the stages of cleavage are easier to observe than in *R. temporaria*. It is not clear whether they realized that the cleavage-furrows (*lignes* or *sillons*) went so deep as actually to divide the egg. They remark that the egg was soon *divisé* into two very pronounced *segmens* (p. 111), but from what they say farther on it is clear that at the stage to which they refer the furrow had not yet reached even the surface of the lower pole of the egg. In general, their observations were remarkably accurate. One of them observed and sketched what was occurring, while the other wrote down a short description. The later stages of cleavage occurred so rapidly that the authors could only compare them to the dissolving views (*changemens à vue*) seen at the theatre. Some of their drawings are here reproduced in fig. 17. They had no idea that the *segmens* were cells.

Prévost and Dumas noted the resemblance of the upper pole of the egg to a raspberry, at a late stage in cleavage (p. 112). As we shall see, later observers repeatedly noted the resemblance of such stages to raspberries, blackberries, or mulberries, in the development of various animals.

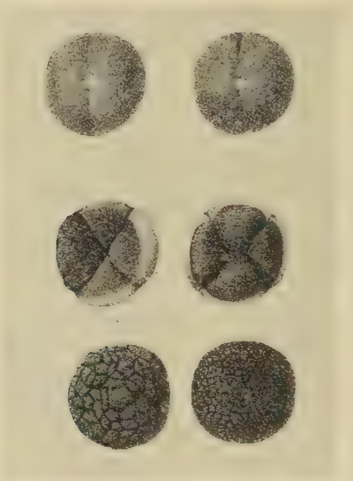


FIG. 17. Some of Prévost's and Dumas's figures of the cleavage of the frog's egg. (Prévost & Dumas, 1824, plate 6, figs. C, D, G′, G″, M, and N.)

Cleavage stages in *Unio* were seen and figured by Carus (1832, pp. 44–45 and his plate 2, figs. I, III, X, XI), but he did not understand the origin or nature of the blastomeres.

It has already been mentioned (p. 415) that de Quatrefages (1834) saw the blastomeres or *globules* of various pulmonate gastropods of fresh water and thought that they multiplied by endogeny. He remarks (p. 109) that in the development of a species of *Limnaea*, the *globules* have formed a *tissu cellulaire* by the sixth day. The meaning of this is unfortunately not clear. We saw in Part I of this series of papers (1948, p. 112) that the expression *tissu cellulaire* was formerly used in a sense that seems very strange to us today, and de Quatrefages does not say distinctly that one globule represents one cell.

The cleavage of the eggs of frogs (*Rana temporaria* and *R. esculenta*) was re-investigated by von Baer (1834). The great embryologist thought that Prévost and Dumas had regarded the process as one of mere furrowing without actual division (von Baer, 1834, p. 481; 1835, p. 6). He made it perfectly clear that in fact the furrows actually divide the egg into discontinuous parts that are only pressed against one another (1834, p. 487). He used the word *Theilung* to describe the process. He compared the embryo at one stage to a blackberry and at a later stage to a raspberry (p. 493). He was thus the first to note the resemblance that was later perpetuated in the word *morula*. (The Romans used mōrum for both blackberry and mulberry. The diminutive form *morula* is a modern invention. The Latin word *mŏrula* meant a short delay.)

Rusconi gave excellent figures of cleavage in the *Wassersalamander* (1836*a*, his plate VIII, figs. 1–8) and of partial cleavage in the cyprinid fishes, *Tinca* and *Alburnus* (1836*b*, his plate XIII).

The cleavage of the egg by the formation of furrows had so far only been observed in vertebrates. Von Siebold now reported the same process in several genera of nematodes (1837). He called the blastomeres *Dottertheile* and remarked that when they had become small through repeated cleavage, the embryo resembled a blackberry. He saw the nuclei in the blastomeres from the 6-cell stage onwards; he did not use the word nuclei, but generally called them *helle Flecke* (p. 212). He called the nucleus of the egg the *Keimbläschen* or *Purkinjesche Bläschen*, and noticed the presence in it of a nucleolus or *Keimfleck* (p. 209). These observations constituted a considerable step towards the recognition of blastomeres as cells.

Schwann regarded *Dotterkugeln* as cells (1838*b*), but it is not clear from the context whether he here refers to blastomeres or yolk-globules. He recognized the protoplasts of the blastoderm of the hen's egg during the first day of incubation as nucleated cells, and gave a good figure of them, showing the nuclei and nucleoli (1839, pp. 63–66, and plate II, fig. 6).

It has been supposed that Cruikshank (1797) may have seen cleavage-stages in the rabbit, but neither his description nor his figures support this opinion. Jones may possibly have seen a morula-stage in the development of the same animal, but cells cannot be clearly seen in his illustration (1837, his plate XVI, fig. 1), nor does he describe them. The first person to describe the blastomeres of a mammal was the Scottish physician, Barry, who had worked with Schwann in Berlin (see Barry, 1838, p. 302). Barry (1839) gave admirable illustrations of cleavage-stages in the rabbit; two of them are here shown in fig. 18. As we have seen (p. 417), he was mistaken about the way in which blastomeres multiply, but credit is due to him for the first clear recognition that blastomeres are cells, or 'vesicles', to use his own word; he equates his vesicles with the cells of Schleiden and Schwann (p. 360). He remarks of the blastomeres of the 4-cell stage, 'Some of these vesicles presented in their interior a minute pellucid space, which may possibly have been a nucleus' (p. 323). In a footnote he adds, 'Later observations strengthen this supposition, and enable me to extend it to vesicles in the succeeding stages. The nucleus was very distinct in each of the two vesicles occupying the centre of the ovum in fig. 105½' (see fig. 18 in the present paper). He remarks, 'The nature of the alterations which the germ undergoes immediately after the termination of the primitive changes now referred to, I do not know, not having carried my investigations beyond that period. It is probable that they consist chiefly in the formation of new vesicles' (p. 365). In a later paper (1840, p. 542) he refers to the blastomeres of the 2-cell stage as 'cells'. Barry mentions the resemblance of the embryo at a certain stage to a mulberry (1838, p. 324).

The fact that blastomeres are cells was recognized by Reichert in 1840, independently of Barry. From his study of the frog's egg, Reichert concluded that the blastomeres gave rise to the cells of the adult, and he followed this out for various tissues (1840, pp. 13, 19, 58; 1841, p. 540). As we have seen, however, he was entirely mistaken about the way in which bastomeres multiply (see above, p. 417).

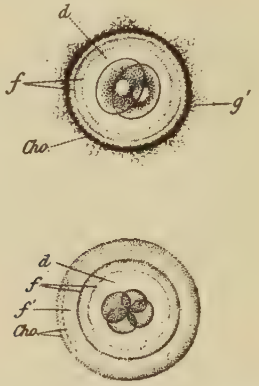


Fig. 18. Barry's figures of the 2- and 4-cell stages in the development of the rabbit. (Barry, 1839, plate VI, figs. 105½ and 106.)

The recognition by Barry and Reichert that blastomeres were cells constituted an important advance.

Bagge (1841) continued the work of Siebold by studying the early development of the eggs of nematodes. He followed cleavage in several species. In his figs. XI–XIX and XXI–XXIII (the latter wrongly labelled XXV–XXVII through the *Molestissima negligentia* of the engraver), he shows clearly, in the species he calls *Ascaris acuminata*, how the size of the cells is reduced by repeated division from the uncloven egg to the worm-shaped embryo. His great merit was his recognition (p. 10) that the large *vitelli partes* or blastomeres gave rise to the little *globuli* of the late embryo, by a process of repeated cleavage. It is unfortunate that when he used the word *cellulae*, he meant nuclei. He saw these in the blastomeres (e.g. in the 6-cell stage of *Strongylus*; see his fig. x). He noted the resemblance of the embryo at one stage to a blackberry or mulberry (one cannot say which, for he writes in Latin).

Bergmann of Göttingen, a student of the development of frogs and newts, was the first person who both understood the nature of cleavage and also recognized blastomeres as cells. He summed up his conclusions in words that show a restraint that is perhaps laudable. 'I may therefore state', he wrote, '*that the cleavage of the amphibian egg is an introduction to cell-formation in the yolk.* Indeed, I would even call it cell-formation, if the first, larger divisions of the yolk could unreservedly be called cells' (1841, p. 98). He compared his findings with those of Mohl in filamentous algae (see above, p. 425). He saw the *hellen Flecke* in the blastomeres, and thought that they might be nuclei.

Bergmann's slight hesitancy was caused by the absence of any resemblance between cleavage and the process of cell-formation as described by Schleiden. Although Bagge had not specifically homologized the *vitelli partes* and *globuli* with cells, yet Bergmann's hesitancy dissolved when he read the former's paper and found his own discoveries repeated in another group of animals. Bergmann now wrote of 'the identity of cleavage and cell-formation' (1842, p. 95). His two papers should form a landmark in the history of the cell-theory.

Vogt was one of the first to admit that Bergmann might be right, so far as frogs were concerned, but he denied that the latter's ideas were applicable to *Alytes* (1842, pp. 9, 25). Rathke (1842) recognized the blastomeres of *Limnaea* as cells: he saw within each its nucleus (*Kern*), and within the latter its nucleolus (*Kernkörper*). Bischoff (1842) gave excellent figures of cleavage in the rabbit (his plate III, figs. 21–26), but denied that the blastomeres of this animal were cells (p. 79). He was influenced mainly by the absence of cell-walls.

The truth now began gradually to be accepted. Reichert withdrew (rather half-heartedly) his idea that cleavage merely separated blastomeres that had already been formed endogenously, and allowed that it was the 'first act' of cell-formation (1846, pp. 274, 278). Kölliker was more explicit in his change of opinion. He made the important generalization that blastomeres always multiply by division, like infusoria, never by endogeny, as Reichert had supposed (Kölliker, 1847, pp. 12–13). Nearly a quarter of a century before, Prévost and Dumas had given a clear description of the cleavage of the frog's egg; but in the intervening years the theory of endogeny had taken root so firmly, that when blastomeres began to be regarded as cells, it was found hard to believe that they multiplied by division. Kölliker's generalization marked the end of the controversy on this subject. The credit, however, belongs to Barry, Reichert, Bagge, and especially Bergmann. Weldon (1898) was wrong in giving it to Kölliker.

The multiplication of other kinds of cells by division

Although the study of protists, filamentous algae, and blastomeres was of paramount importance for the discovery that cells multiply by division, yet quite a number of relevant observations were made from time to time on other objects. Grew may have had cell-division in mind when he remarked that some of the 'Pores' of the pith of plants were 'divided with cross Membranes' (1672; see above, p. 409); and Wolff, though he believed in exogeny as the usual method of cell-formation, yet allowed that partitions or *dissepimenta* were sometimes formed across the large cells of plants, with the production of smaller, included cells (1759, p. 21).

The division of the pollen mother-cells of *Cobaea scandens* (Polemoniaceae) into four young pollen-grains was observed by Brongniart (1827). His illustrations are here reproduced in fig. 19. The granules contained in the mother-cell, instead of forming a single mass, 'reunite in four perfectly distinct spherical masses, which float freely in the interior of the transparent utricle that contain them'. Each of these spherical masses 'continues to grow, and the membrane that covers it soon takes on a cellular aspect; the distended utricles that contain these globules in groups of four, split open' (p. 27). It is to be noticed that Brongniart did not describe binary cell-division.

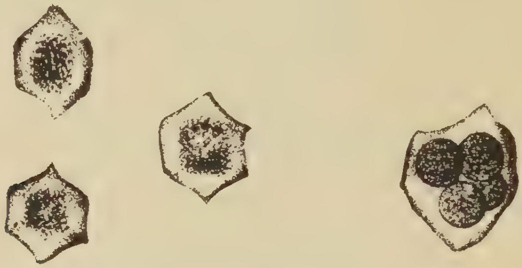


Fig. 19. Brongniart's figures of the division of the pollen mother-cells of *Cobaea scandens*. (Brongniart, 1827, plate 34, fig. 2 (E, F).)

Dumortier stated that all cells that are arranged in *rows* in the fronds of algae and in fungi, mosses, and Jungermanniales, multiply by partitioning in the same way as the cells of filamentous algae (1832, p. 229). Meyen, who was acquainted with Dumortier's findings, observed cell-division by partitioning in the developing lateral axes of *Chara*, and claimed to have observed it also in moulds (1838, pp. 339, 345). He would not use the name *Theilung* for those cases (e.g. endosperm-formation) in which new cell-walls are formed over the whole of the surface of the newly produced protoplasts; he referred to it instead as *Bildung der Zellen in Mutterzellen* (p. 346). Mirbel's *développement intra-utriculaire* (1835, p. 369) may perhaps have been cell-division. He seems to have recognized the varieties of the process that are called in the present paper 'partitioning' and 'cell-division with formation of entirely new cell-walls'.

Schwann himself allowed the possibility that in certain cases new cells might arise by partitioning of pre-existing cells (1839, pp. 5, 218), but he does not appear to have made any actual observations on this subject. Schleiden also equivocated slightly in a book (1842) published four years after his original communication. Though he still regarded endogeny as the standard method by which new cells arise in plants, and questioned the accuracy of Mohl's and Meyen's accounts of cell-division, yet he was clearly puzzled by observations he had made on the parenchyma of certain unspecified cacti. The cells were very regularly arranged, and he noticed here and there that one of them, though appearing single by its relation to the others, yet was clearly divided in two by a partition. This was very suggestive of multiplication by division; but Schleiden often noticed a *Cytoblast* on each side of the partition, and this allowed him to think it probable that even in this case, his own particular form of endogeny had been at work (pp. 267–9).

Remak (1841) would appear to have been the first person to observe a stage in cell-division in a many-celled animal, apart from cleavage. In the blood of a chick-embryo, in the third week of incubation, he observed pear-shaped cells, joined together in pairs by the stalks; each cell had a nucleus. He seems to have seen the remains of the spindle in the bridge between the two cells. He interpreted what he saw as a stage in cell-multiplication by division.

Vogt's observations on the notochord of the newt, *Triturus*, published the next year, were much more complete. He examined the cells at successive stages of larval development. 'The cell-wall bends inwards', he says, 'constricts, and thus at last divides into two halves, which are both exactly similar to one another and to the previously undivided cell, and they both continue their independent lives as cells' (1842, p. 128, and his plate 2, figs. 15 and 16; see also his pp. 46–47). It is strange that this clear description of the multiplication of cells by division should have been written by one who thought that cells ordinarily arise exogenously in a *Cytoblasteme* (see above, p. 412).

Valentin thought that blood-cells and other separate cells of multicellular animals in some cases multiplied by division (1842, p. 630). He instances the cells (*kernartigen Körperchen*) of the thymus gland of the embryo of the sheep; his figure (plate V, fig. 65) may indeed show actual stages of cell-division.

One of the most important contributors to our knowledge of cell-division was Nägeli (1844, 1846), but curiously enough, he himself would not allow that most of the processes he was studying constituted cell-division. He restricted the idea of division to *Abschnürung*, that is to say, to what is called in the present paper 'cell-division with constriction of the cell-wall'. In his earlier paper (1844, p. 97) he denied that this process was ever complete: a partition appeared before the constriction had become very deep. He writes of 'so-called' cell-division (p. 110). Later, however, he allowed the reality of complete *Abschnürung* in certain cases (1846, p. 60). By 'cell-formation' he meant the formation of cell-walls. For him, the cell-wall *was* the cell: if it did not constrict to nip the pre-existing cell in two, cell-division did not occur.

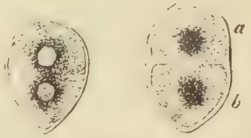


FIG. 20. Nägeli's figure of the division of a germinating spore of *Padina* (Phaeophyceae). (Nägeli, 1844, plate II, figs. 4 and 5.)

Although he concentrated so much attention on the wall of the cell, Nägeli by no means overlooked its *Inhalt* or protoplasm, and indeed he made some interesting observations on the multiplication of nuclei, to which it will be necessary to refer in the next paper in this series. He noticed that when cell-multiplication is about to occur, there is first of all an isolation or individualization of parts of the *Inhalt* (or, in modern terms, the protoplasm divides into two or more parts) (see fig. 20). A *Membran* or thin cell-wall then forms round each of the parts. In *wandständige Zellenbildung* this new cell-wall, from the moment of its appearance, is everywhere in contact with the wall of the original cell, except where the new *Inhaltspartien* are divided from one another; here a new partition is formed. This is a kind of cell-division by partitioning, in the terminology used in the present paper. In *freie Zellenbildung* the *Inhaltspartien* separate themselves entirely so that each is 'free'; a new wall is formed round each, and these walls are nowhere in contact with the original cell-wall (Nägeli, 1846, pp. 51, 60, 62; see p. 420 of the present paper).

The strangeness of Nägeli's papers is mainly verbal. When once one has grasped his use of words, it is clear that he made a massive contribution to our knowledge of the process by which cells multiply. He studied it in all the major groups of plants (other than bacteria). From his time onwards scarcely anyone could take seriously the contention that plant-cells multiply exogenously, and the foundation had been laid for a general understanding that the protoplast multiplies by division.

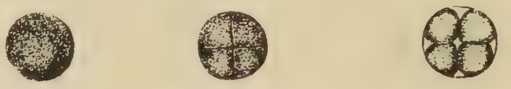


FIG. 21. Reichert's figures of the division of the primary spermatocyte of *Strongylus auricularis* into 4 spermatids. (Reichert, 1847, plate VI, figs. 5–7.)

It will be remembered that Reichert was mistaken about the nature of cleavage (see above, p. 417). He made important observations, however, on the multiplication of the male germ-cells of the nematode *Strongylus* (1847). He identified the cells at the blind upper end of the tubular testis as 'elementary nucleated cells', and understood that a series of stages in spermatogenesis was displayed along the tube, the pear-shaped ripe spermatozoa being found at the opposite end. Working along the tube from the blind end, he saw stages in the division of the spermatogonia (pp. 101–2), and further along again he saw and figured what were evidently the meiotic divisions (pp. 110–14); these he compared with the processes of pollen-formation (which, as we have seen, had been observed by Brongniart). Some of his figures are reproduced here in fig. 21. Like Nägeli, Reichert could not escape from the ideas of the nature of the cell that were current in his time, and he described cell-division as *Zellenbildung um Inhaltsportionen*. The process whereby the *Inhalt* (protoplasm) divided into its *Portionen* (daughter-protoplasts) was evidently of secondary interest to him, for he was always looking for the formation of a *Zelle* (cell-wall) round a newly formed protoplast. Like Nägeli again, however, he by no means overlooked the *Inhalt*, and it will be necessary to revert to his work on *Strongylus* in the next paper in this series, in which the multiplication of nuclei will be considered.

Omnis cellula e cellula

'The origin of cells', wrote de Candolle in 1827, 'like everything connected with the origin of organisms, is a problem that it is absolutely impossible to resolve in the present state of knowledge' (p. 27). Twenty years later the main facts had been discovered. Morren had recognized *Crucigenia* as a cell and followed its multiplication by division. Dumortier, Mohl, and Meyen had discovered how the cells of filamentous algae multiply. Dumortier and especially Nägeli (despite his misleading terminology) had established cell-division as the standard method of cell-multiplication in the main groups of plants. That the cleavage of eggs is cell-division had been revealed by the investigations of von Siebold, Barry, Reichert, Bagge, and Bergmann. Finally, cell-division had been demonstrated in notochordal and spermatogenetic tissues by Vogt and Reichert. It remained to realize that the process that had been studied and reported over and over again was the universal method by which cells multiply.

This realization, epitomized in the words *Omnis cellula e cellula* (surely one of the grandest inductions of biology), we owe almost entirely to two men, Remak and Virchow. The Latin phrase, however, does not exclude the origin of new cells by endogeny, and it must be remarked in passing that Raspail, Turpin, Schleiden, and Goodsir would perhaps have assented to it, if it had been put forward in their time. In fact, however, the phrase was introduced solely in reference to the origin of new cells by *division*.

Remak and Virchow had both been pupils of Johannes Müller, both were practical medical men as well as biologists, and both were in their thirties. In other respects they were very different. Remak was the typical research-worker. He carried out thorough investigations in the laboratory; he studied carefully the work of others, and made full acknowledgement of it; he wrote in a straightforward style and eschewed all fanciful ideas. Virchow, on the contrary, soared away in the manner of his predecessors in the school of *Naturphilosophie*, and left the reader guessing what the actual facts were that led him to his conclusion, and who discovered them.

Although both men published in 1852 and their papers cannot be exactly dated, yet the circumstantial evidence suggests that Remak was the first in the field. It will be remembered that he had observed a stage in the multiplication of blood-corpuscles by division in 1841 (see p. 433). In 1851, when writing on the cleavage of the frog's egg, he said that he must reserve to another paper his remarks on the transition from the cleavage-cells to the tissues, by repeated cell-division (p. 496). The promised article appeared in the following year.

By a careful review of the available evidence, but without adding new observations, Remak (1852) set out to explode Schwann's idea of the exogenous origin of cells and to set up in its stead a general theory of their multiplication by division. He remarks that the botanists no longer believe that cells arise outside pre-existing cells. To him, the extra-cellular origin of cells is as unlikely as the *Generatio aequivoca* of organisms (p. 49). Between the cleavage-cells there is no intercellular substance in which new cells could originate exogenously. Remak breaks loose at last from the domination of the vitelline membrane, which had so misled previous investigators. For them, the vitelline membrane *was* the cell; the protoplasm was its *Inhalt*. Thus, since the vitelline membrane did not change during cleavage, cell-division did not occur!

Remak plays down the *Dotterhaut*, remarking that it does not participate in the formation of the egg-cell. The protoplasm of the egg-cell passes over into that of the embryonic cells, and the nuclei of the latter are the derivatives of the nucleus of the first cell. Remak thinks it unlikely that new cells arise from extracellular substance even in diseased tissues. 'The statement that the cells of animals, like those of plants, have only an *intracellular* origin, seems to me to be a proposition established by a long series of reliable experiences' (p. 55).

Remak's elaborate study of the development of the chick and frog, recorded in his celebrated book (1855), convinced him of the correctness of the views he had expressed in 1852. Towards the end of the work, in a valuable general review of the cell-theory, he upheld division (*Theilung*) as the standard method of cell-formation. He is concerned once again by that bugbear of the cytologists of his time, the vitelline membrane, and points out that it is not always possible to distinguish with certainty between cell-membranes, thickenings of the outer parts of cells, and intercellular material (p. 174). He realizes that what is essential is the protoplasm, and this *divides*; 'all animal cells arise from the embryonic cells by progressive division' (p. 178). He is puzzled, however, by the fact that in certain rhabdocoels the germinal vesicles are formed in one organ, and the yolk in another; this makes him hesitate about saying unequivocally that the ordinary egg, with its yolk, is a cell. He understands clearly that the division of the egg by cleavage-furrows is not always complete.

It is impossible to tell whether Virchow had read Remak's paper of 1852 when he published his own in the same year. He makes no mention of Remak; but this is perhaps not very significant, for he mentions no one who had written on the multiplication of cells except Schleiden, Schwann, and Kölliker (and the latter only in connexion with the contractility of the vessels of the umbilical cord!). One does not know what were the chief facts that convinced him of the origin of cells from pre-existent cells by *Zertheilungen und Zerspaltungen* (1852, p. 377). Somewhat understating the case, he admits that his earlier definition of the cell as 'the indivisible living One' (see above, p. 419) was *nicht ganz richtig*; for *überall findet sich das Princip der Theilbarkeit, der Spaltbarkeit* (p. 378).

Like Remak, he returned to the theme in 1855. Like Remak, he pointed out that if cells did not arise from pre-existent cells, the state of affairs would resemble *Generatio aequivoca*. Unlike Remak, he uses strangely violent language in denouncing spontaneous generation as 'either pure heresy or the work of the devil'. He then proceeds to the great generalization. 'I formulate the doctrine of pathological generation, of neoplasia in the sense of cellular pathology, simply thus: *Omnis cellula a cellula*' (p. 23). It is noteworthy that in coining the aphorism, Virchow applies it to diseased tissues, and takes for granted that it applies to normal cells. It seems to have been Leydig who first put the phrase in its final form. Like Remak and Virchow, Leydig first of all denies the reality of *generatio aequivoca*. 'Observation knows only an *increase of cells from themselves*,' he proceeds, 'and the same validity might be ascribed to the proposition *omnis cellula e cellula* as to *omne vivum e vivo*.' This was the orm of words adopted by Virchow in his statement on the subject in the *ellularpathologie* (1859, p. 25). 'Just as we no longer allow', he wrote, 'that a oundworm originates from mucous slime, or that an infusorian or a fungus or n alga forms itself from the decomposing remains of an animal or plant, so lso we do not admit in physiological or pathological histology that a new cell an build itself up from a non-cellular substance. Wherever a cell originates, n that place there must have been a cell before (*Omnis cellula e cellula*), just s an animal can only originate from an animal and a plant from a plant.'

If we deliberately overlook the strange style in which Virchow wrote his aper of 1852, we can see that he made a contribution towards the understanding that cells are derived from pre-existing cells by division. But if we ompare his writings with Remak's, we cannot fail to recognize that the latter's nust have had far more influence. Indeed, it does not seem likely that Virchow's vritings by themselves would have had much effect upon opinion. Remak's aper of 1852 contains no catch-phrase, but it stands out as the first clear and olidly backed general statement of the way in which cells multiply.

It must be regretted that in later years Remak (1862) withdrew to some xtent from the position he had adopted. No exception was definitely known o the rule that in normal tissues, cells multiplied by division: that he still llowed. But he now maintained that endogeny occurred in diseased tissues, nd that in this process, pre-existing nuclei were not concerned. Further, ne thought it probable that certain cells in normal tissues multiplied endogenously. He considered that spermatozoa originated within a mother-cell, and that merogony was a form of endogeny. The nuclei of certain cells, in his view, could not be traced back to the nuclei of the embryo. He instanced the star-shaped cells of connective tissue (presumably the fibroblasts), and the cells of the cutis and of the smaller branches of the blood-vessels of the frog.

These doubts must not be allowed to obscure the service that Remak had rendered to biology at the appropriate moment, ten years before. Nevertheless, we must guard against overestimating his contribution. Important though he was, Remak was not the discoverer of the way in which cells multiply. Trembley, Morren, Ehrenberg, Dumortier, Mohl, Meyen, Prévost, Dumas, von Siebold, Barry, Reichert, Bagge, Bergmann, Nägeli—these were the men whose discoveries had produced a situation in which a great generalization would be acceptable. Remak supplied it.

ACKNOWLEDGEMENTS

I am particularly grateful to Dr. Charles Singer for calling my attention to two important papers that I should otherwise have missed. I take the opportunity of adding Klein's *Histoire des origines de la théorie cellulaire* (1936) to the list of valuable works on the subject, given in the first part of this series of papers. My work on the cell-theory has continued to receive the support of Professor A. C. Hardy, F.R.S.

Correction. In part III of this series of papers (1952), I suggested that the word *coenocyte* appeared to be superfluous. Further consideration has led me to change this opinion. It seems desirable to have a special name for those syncytia that resemble single cells in shape (e.g. the binucleate components of mammalian liver). The word coenocyte will therefore be used in future parts of this series of papers when it is necessary to specify this particular category of syncytia.

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On the Origin of the Metazoa

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SUMMARY

A new view is expressed regarding the possible evolution of the Metazoa from unspecialized and relatively simple Metaphyta, instead of from colonial or partitioned Protozoa.

THIS is a short paper to present a very simple idea regarding the origin of the Metazoa, one which, as far as I am aware, has not been expressed before. It is in a sense a corollary to the views expressed by my old friend and colleague Dr. J. R. Baker in his article on 'the Status of the Protozoa' (1948); indeed, the idea owes its origin to that article for it occurred to me after reading it.

Baker, it will be remembered, defines a cell as 'a mass of protoplasm, largely or completely bounded by a membrane, and containing within it a single nucleus formed by the telophase transformation of a haploid or diploid set of anaphase chromosomes'. He argues that while some Protozoa, such as the Ciliophora and certain Radiolaria, are not cells in the sense of this definition, there are many which can properly be regarded as unicellular organisms. He stresses that there is no fundamental difference between the cleavage of an egg and the multiplication of a unicellular protozoon by division. At the end of his article he discusses the evolution of Metazoa from Protozoa. It is well known that there are two schools of thought regarding this question. On the one hand, there are those who hold that the Metazoa were derived from a group of protozoan cells which had failed to separate after repeated division, i.e. from a colony of cells which have gained a new and corporate individuality; on the other hand, there are those who believe that the origin of the many-celled condition was due to the development of partitions within a multinucleate protozoon. Baker discusses both sides of the controversy and points out how it is more difficult for the zoologist to imagine the evolution of a metazoon from a protozoan colony than it is for the botanist to imagine a similar step in the evolution of multicellular plants. Since my suggestion springs directly from his argument of this point, I will, with his kind permission, quote him at some length. He writes as follows:

> 'It may be remarked that while such forms as *Volvox* raise a storm of controversy among zoologists, they are not regarded as unusual by botanists, who are familiar with such colonial forms in groups other than the phytomonads. Botanists regard them as composed of cells, which are comparable on one hand with the individuals of what they call unicellular species, and on the other with the cells of higher but related forms, in which there is somatic differentiation (heterotrichous forms and other algal metaphytes).

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> 'If we seek the reason why zoologists are more divided in this controversy than botanists, we shall surely find it in a fundamental difference between plants and animals. In the lower plants *each group* tends to present both unicellular and metaphytic representatives, and botanists are repeatedly confronted with the intermediate forms. Metaphytes, in fact, have obviously originated independently many times, so that no one would propose the word "Metaphyta" in any classification that was supposed to be based on evolutionary principles. With animals it is far different. Zoologists have not got intermediates again and again before their eyes. In the great group of Rhizopods, for example, there is no example of a multicellular form (the word "cell" being here used as defined above). It would not appear that anyone has discussed the reason for this striking difference between plants and animals. The following suggestion may be made. The unicellular plant absorbs nutriment from all sides equally, and when, in the course of ontogeny or phylogeny, it becomes a metaphyte, there is no fundamental change in this respect: a cell divides without separation and the two products continue to absorb nutriment over most of their surfaces. The passage from the unicellular form to the metaphyte is therefore easy. In the case of animals, however, there is an important change when a unicellular form becomes a metazoon: a *new method* of feeding must be adopted. We see this particularly clearly in the ontogeny of *Dendrocoelum*. The blastomeres feed at first saprozoically, but at last a profound change occurs: a set of digestive organs originates, and a new method of nutrition begins. Most animals overcome this difficulty in ontogeny by placing enough reserve food-stuffs within the single-celled stage to make feeding unnecessary. It is easy to see what evolutionary difficulties must be presented when a *new system* of nutrition must be acquired before advance can occur. The difficulties would be greatest when a protozoon had a localized mouth. If the products of division of such an animal were to adhere together and each were to acquire its own mouth, no advance would be made towards the evolution of a metazoan alimentary canal. This suggests that the Metazoa may have arisen from primitive Protozoa, unprovided with localized organs of assimilation. These considerations seem to make it clear why intermediates between unicellular animals and Metazoa are rare, while intermediates between unicellular plants and metaphytes are common. (The colonial phytomonads are, of course, only regarded as animals by zoologists on account of their motility: their nutrition, it need scarcely be said, is holophytic or saprophytic.)'

We are concerned here with the origin of the true Metazoa, leaving on one side the sponges, sometimes called the Parazoa, which with their more limited integration may well have been quite independently derived from aggregations of choanoflagellate-like protozoa. The simple idea I want to suggest is that the Metazoa have not been derived from the Protozoa at all, but from relatively simple metaphytes which, after they had evolved from protophytes began, perhaps as a result of a shortage of phosphates and nitrates, to capture and feed upon other small organisms as do the higher insectivorous plants. Among the Protista it is clear that animals have evolved from plants more than once; in several different groups of Protophyta, as Fritsch (1935) has so well shown, we can arrange parallel series passing from holophytic forms at one end to holozoic forms at the other. In addition to such changes among the flagellates of the class Euglenineae, Fritsch points out and compares these parallel volutionary lines in four different classes of the simpler algae: Chlorophyceae, Xanthophyceae, Chrysophyceae, and Dinophyceae. If animals have evolved rom plants several times at the unicellular level, is it not possible that animals might, at least once, have been so derived from plants at a not too specialized multicellular level? This suggestion may perhaps be worth bearing in mind because it does get over the very real difficulty which Baker has pointed out, hat of how a metazoon could have evolved from a colony of protozoa, each, as ndividuals, already adapted for the capture of prey.

It would be unprofitable to elaborate the idea in much detail. The many different devices evolved by the various carnivorous plants to enable them to secure their food suggest how such a metazoan organism may have been derived. It is not difficult to imagine a spherical volvox-like metaphyte developng a little pocket-like invagination in which small protozoa or protophyta might collect, die, and provide breakdown products to be absorbed by the cells lining the cavity. Such little pockets would be analogous with the bladders of *Utricularia* which capture water-fleas. How similar in general action, although different in physiological detail, are the 'tentacles' which capture flies on the leaf of the sundew *Drosera* to those which capture crustacea on the polyps of a hydroid! The gradual transition from a simple metaphyte to a simple polyp-like metazoon—a bladder-like cavity with tentacles—seems no more difficult to conceive than the evolution of the higher animal-like insectivorous plants; we have only to imagine the process going so far as to cut out all photosynthesis, thus making the organism holozoic—as indeed has occurred repeatedly at the unicellular level of the flagellates and algae.

If the Metazoa should have evolved from Metaphyta then the findings of Gohar (1940) are of special interest; he claims to have shown that Alcyonarians of the family Xeniidae feed entirely upon the photosynthetic products of their symbiotic algae and never capture animals for food at all; by a circuitous route they appear now to be metazoa which are on their way back to becoming metaphytes and must, if the whole colony is to grow, be taking in nutritive salts from outside for the growth of their enclosed plant cells. (It is perhaps just possible, but most unlikely, that the zooxanthellae of the Anthozoa, which cannot be cultured outside their 'host', might be plant cells remaining as a legacy from the original metaphytic days and not separate symbiotic algae as in other such associations.)

I do not wish here to enter into the controversy between the former two opposing views as to the origin of the Metazoa; the sole purpose of this short communication is to state a third possible view for consideration. I am indebted to Dr. J. R. Baker not only for his article which gave rise to this idea, but for his interest in the suggestion and his discussion of it.

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Nuclear Transplantation in Amoebae. I.

Some species characters of *Amoeba proteus* and *Amoeba discoides*

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SUMMARY

1. The object of this work was to find characteristic differences between stock cultures of *A. proteus* and *A. discoides* in order to assess results obtained in experiments involving nuclear transplantation.

2. Previous work on the two species was not found to yield satisfactory diagnostic features.

3. Methods of establishing and maintaining clones and of observing living amoebae are described.

4. As a result of the study of outline drawings of active amoebae and of measurements of the largest diameter of the nuclei, two characters were found which enabled us to distinguish cultures of *A. proteus* and *A. discoides*. They were (*a*) the pattern of locomotion and (*b*) the distribution of nuclear diameters within a culture.

5. The outlines of active *A. discoides* tend to be more serrated than those of *A. proteus*. Outline drawings were readily sorted by independent observers into two categories corresponding fairly closely to the two species. By this method healthy cultures can be identified. Individual amoebae are not always identifiable.

6. An analysis of the longest nuclear diameters of amoebae of the two species showed that the average of a clone and the distribution of nuclear diameters within a clone are typical for each species and are little affected by the age and state of nutrition of the culture. The average nuclear diameter in our cultures, which was found to be independent of the nuclear size of the parent amoeba, was 45 μ (s. d. 5·6) for *A. proteus* and 38·2 μ (s.d. 3·6) for *A. discoides*.

7. The possibility is discussed of using physiological and serological, rather than morphological properties to distinguish between the species.

INTRODUCTION

THE classification of the amoebae has been a subject of controversy for almost two centuries. It is not the object of this paper to go into the taxonomy of the Amoebina (for review see Leidy, 1878 and Schaeffer, 1916 and 1926) nor to discuss the thorny problem of what constitutes a species among asexually reproducing Protista. In other publications (Lorch and Danielli, 1950, 1953), experiments on transferring the nucleus of one species of amoeba (*A. proteus*) into the cytoplasm of another species of amoeba (*A. discoides*) are described. The object of these experiments was to study the respective roles of nucleus and cytoplasm in maintaining the degree of differentiation characteristic for the species used. It was therefore necessary to find characteristic differences between the two species.

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Before 1916 authors used the term ‘*A. proteus*’ for any of the large, monc nucleate freshwater amoebae. Schaeffer showed, by means of isolation pedigree that this group could be split into three types of amoebae with distinct char acters which bred true to type. Since ‘the best test of a species is in the breed ing of it’, Schaeffer felt justified in placing the three amoebae in separat species which he called *A. proteus*, *A. discoides*, and *A. dubia*. He later (192 went so far as to create separate genera and called the three amoebae *Chao diffluens*, *Metachaos discoides*, and *Polychaos dubia* respectively. But mos authors have adhered to the first classification and we shall use Schaeffer’ 1916 nomenclature here. The appearance of an amoeba is very dependent o the environment of the animal before and during the time of observation. Th fact that the free-living amoebae are always changing—not only their shap but also the very consistency of their cytoplasm—makes accurate descriptio of the species difficult. Schaeffer bases his differential diagnosis chiefly on th following characters:

1. Mode of formation of the pseudopods, which is dependent on th physical properties of the cytoplasm.
2. Size of the animal.
3. Shape and size of the crystals in the cytoplasm.
4. Shape and size of the nucleus.

To this may be added the following features which, if carefully studied, migh reveal constant differences between *A. proteus* and *A. discoides*:

5. The pattern of locomotion.
6. The appearance of the animal during binary fission.
7. The mitotic cycle of the nucleus.
8. The life history.
9. Physiological properties.
10. Serological properties.

Keeping these ten points in mind, a thorough study of *A. proteus* an *A. discoides* was made over a period of 3 years. In the present paper the result of this study will be described and related to the more recent literature.

MATERIAL

Stock cultures of amoebae were kindly supplied by Sister Monica Taylo and Sister Carmela Hayes of Notre Dame Training College, Glasgow. Th cultures were referred to as *Amoeba proteus* and *Amoeba discoides*. Clones wer established from both species, and all observations and experiments wer carried out on mass cultures grown from single amoebae.

METHODS

Culture methods

Various methods of raising clone cultures were tried. The following wa found most successful: single active amoebae were picked out of a culture dish by means of a mouth pipette and distributed in solid watch-glasses containing 2 ml. of a 'food culture' of ciliates or flagellates, or a mixture of both in the following modification of Chalkley's (1930) medium:

Stock solution

NaCl	16·0 gm.
$NaHCO_3$	0·8 gm.
KCl	0·4 gm.
$Na_2HPO_4.12H_2O$	0·2 gm.
Water (glass-distilled)	1000 ml.

To make 1,000 ml. of culture medium add 5 ml. of stock solution to 995 ml. of glass-distilled water. The pH is about 7·5.

The success of the experiment depends on the food culture. The most suitable food organisms were found to be the flagellate *Chilomonas paramecium* and the ciliate *Colpidium* sp.

The food culture in the watch-glasses was sucked out and replaced by fresh culture every 4–7 days, depending on the rate of multiplication of the amoebae and on bacterial growth. Amoebae do not thrive when dense bacterial growth is present.

When the number of amoebae in a solid watch-glass exceeded 30 (i.e. after 10–14 days) the contents were transferred to a Petri dish containing food culture. Here the amoebae may be left for 3–4 weeks, the food organisms being replenished if necessary. After this time the culture was usually well established and ready for transfer to the standard culture dish. This is a shallow pyrex dish, 10 cm. internal diameter, filled with 100 ml. of Chalkley's medium to which 4 boiled wheat grains were added. The culture dishes were kept covered, in dim light, at room temperature (18–23° C). Every 4 weeks the cultures were examined under a dissecting microscope, most of the fluid and 2 of the wheat grains were sucked out, and fresh Chalkley's medium and 2 boiled wheat grains were added. Every 2 or 3 months the cultures were transferred to clean dishes. There is considerable variation in the state of amoeba cultures even when established under relatively standard conditions; therefore the treatment of the mass cultures was according to need rather than rigidly standardized. In some dishes amoebae multiplied rapidly. In these cases the food organisms have to be replenished from time to time. In other dishes there appeared dense bacterial growth which led to a rapid increase in the ciliate population and a decline in the number of amoebae. The latter became sluggish and were attacked and eaten by the 'food' organisms. The cultures were always kept free from rotifers, worms and Crustacea. Algae do not appear to be harmful but were excluded because in their presence it is difficult to see the amoebae clearly. Various moulds were noted on the wheat grains. They cannot be avoided in non-sterile cultures and are certainly not harmful. The mould hyphae are often densely covered with amoebae whose extended pseudopods form a network enmeshing the ciliates and flagellates which feed on the bacteria accumulating round the decaying wheat.

Cultures may be maintained indefinitely in this manner. Like Dawson (1928) we found that the 'depression periods' described by Taylor (1924) do not occur if a plentiful food supply is maintained and dense bacterial growth avoided.

Methods of examination

The paraffin chamber (Commandon and de Fonbrune, 1938) was found most useful for observing active amoebae over protracted periods of time. The amoebae were placed in very shallow hanging drops on clean coverslips. They remain active in the drops for days but do not divide unless food organisms are supplied and the fluid is renewed periodically. To study the pattern of locomotion of the two species camera lucida drawings of the outlines of active amoebae were made, each animal being drawn at 1-minute intervals for 4–5 minutes. The significance of these outline drawings was estimated by the following method: unlabelled sheets of drawings, each representing 4–6 consecutive sketches of one amoeba of either species, were mixed in batches of about 20 and sorted by various observers. The observers were provided with type specimens considered to be typical *A. proteus* (P) and *A. discoides* (D), and asked to sort the drawings into 4 groups: typical P, like P, like D and typical D. The intermediate groups 'like P' and 'like D' were always included because the sorting method was also used to detect intermediate types in amoebae with transplanted nuclei (Lorch and Danielli, 1953).

In order to compare the nuclear sizes, measurements of the longest diameter of the nuclei (which are discoid in both species) were made with an ocular scale. Some measurements of the total lengths of amoebae when spread out were also made. The cytoplasmic inclusions were studied under an oil immersion objective by transmitted light. Phase contrast was not found to be advantageous. Dividing amoebae are easily recognized in culture dishes as they are roughly spherical and covered with small pseudopods. Such 'division spheres' were also studied in the paraffin chamber. In order to get a sufficient number of dividing amoebae at a given time, culture dishes were placed in the refrigerator (4° C.) overnight, and brought back to room temperature in the morning (Dawson, Kessler, and Silberstein, 1935, 1937). About 6–7 hours after removal from the refrigerator many amoebae entered mitosis. Fixed and stained preparations of active amoebae and of division stages were made by Doljansky (1954).

RESULTS

General appearance

When comparing amoebae it is important to do so under identical or at least closely similar conditions. An 'old' starved *Amoeba proteus* resembles an 'old' starved *Amoeba discoides* much more than it resembles an active 'young' specimen of its own species. (The term 'old' is used throughout this paper to denote an amoeba which has not divided for several days. A 'young' amoeba is one resulting from a recent division.) In well fed cultures the amoebae divide, on the average, every 36 hours. No significant difference in division-rate was noted between the two species under optimum conditions, but *A. discoides* appears to be less affected by adverse conditions, and is therefore easier to culture. Amoebae from rapidly increasing cultures present the following features: in the culture dish they are firmly attached to the glass or to moulds and are not dislodged by gentle shaking of the dish. They either crawl on a surface or have a rosette-like appearance. The latter is the most common shape wherever food organisms are very plentiful. The amoeba then remains sessile in one spot and feeds by extending short, blunt pseudopods, rather like the partly contracted tentacles of a sea anemone. Such amoebae are always packed with food vacuoles. The more rapidly the animals are dividing, the smaller they tend to be. The daughter cells do not move apart but remain attached side by side. In this way whole colonies of 'rosettes' are formed, usually as a ring round a mouldy wheat grain, at the distance of optimum density of food organisms. Aggregates of amoebae in culture dishes always seem to arise in this way, i.e. by multiplication of amoebae which do not move away, and not by chemotactic migration towards the food source.

If such a 'rosette' amoeba was picked out and placed in Chalkley's medium in a dish or on a coverslip, it did not at first stream about. But as the food vacuoles discharged, the amoeba became more active, and after about 24 hours in a dish it had assumed a stellate appearance with pseudopods radiating in all directions. On a coverslip it crept about incessantly and put out many pseudopods. If the amoeba was now returned to a dense culture of ciliates it began feeding at once and soon returned to the rosette shape which later changed into a division sphere. If starvation was continued for days the amoeba became sluggish and opaque, owing to the increased number of crystals and 'spheres' in the cytoplasm. At this stage *A. discoides* sometimes assumed a clavate shape —monopodal with a clear cap at the anterior end. This shape was only very rarely seen in *A. proteus* cultures. Underfed and starved amoebae are generally not attached to any surface and float about in the culture dish.

Comparisons between the two species regarding cytoplasmic inclusions, mode of pseudopod formation and general pattern of locomotion were made on 'active' amoebae, i.e. on animals which were neither overfed (and hence sessile) nor deprived of food for more than 24 hours. Multi-nucleate amoebae, which are seen occasionally in cultures, were not used. Specimens were also compared during the first few hours after division. The general impression gained after prolonged observation of both species was that *A. discoides* tends to put out a greater number of pseudopods than *A. proteus*. This impression led us to a closer analysis of the shapes assumed during active movement, by making camera lucida drawings of single amoebae. The majority of the sheets of outline drawings, each sheet representing 4 to 6 consecutive drawings of a single active amoeba, were readily sorted by independent observers, whether these were familiar with amoebae or not, into two categories which represented the two species. Examples of outline drawings are given in fig. 1. As described under 'Methods' the observers were supplied with two type-

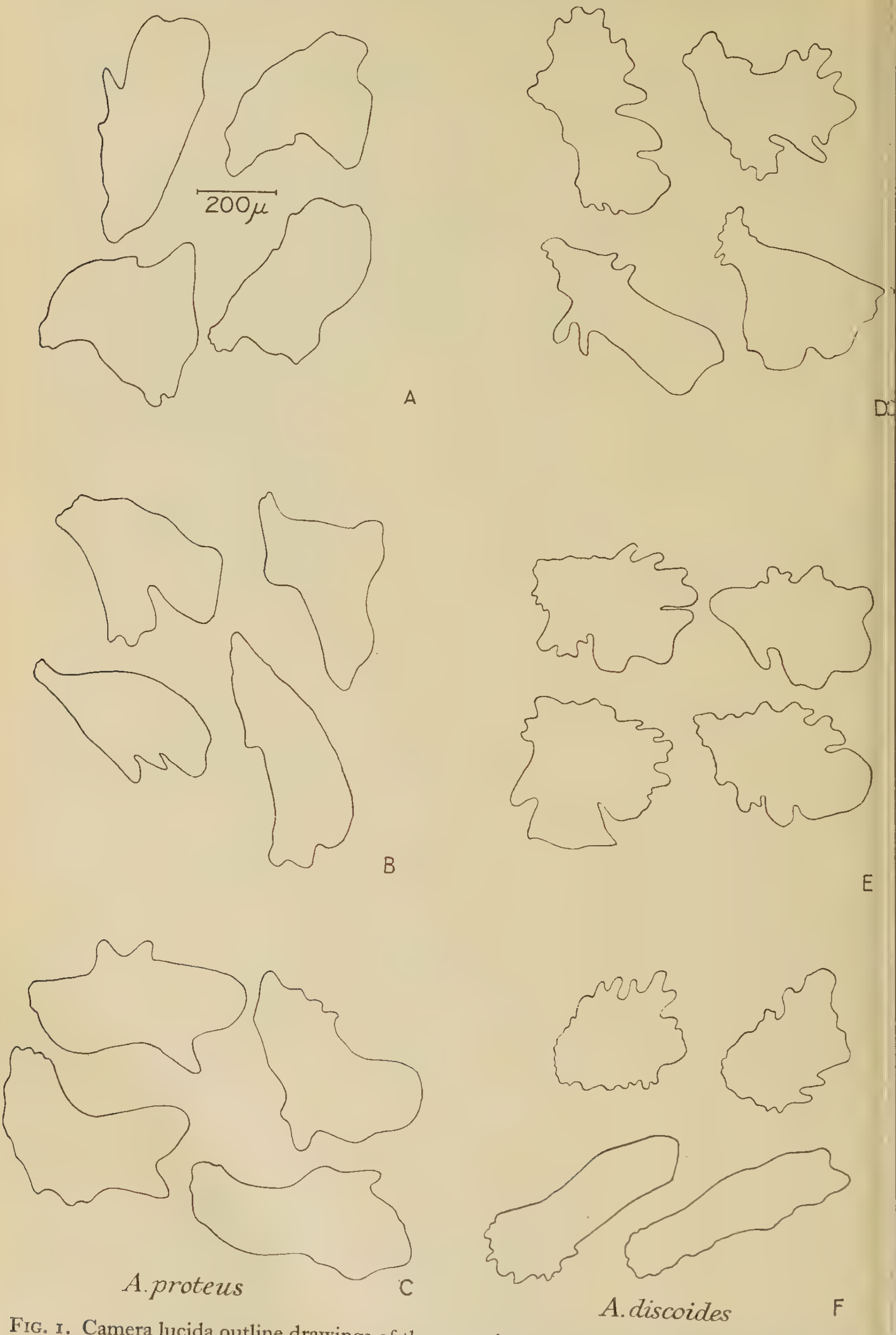

FIG. 1. Camera lucida outline drawings of three specimens of *Amoeba proteus* (A–C) and three specimens of *A. discoides* (D–F). Each amoeba was drawn four times at 1-minute intervals.

specimens and were asked to place the unlabelled drawings into four groups. The number of drawings placed in each group was recorded. Fig. 2 shows a graphical representation of the sorting of drawings of 18 specimens of *A. proteus* and 17 of *A. discoides*. The drawings were sorted five times on different days by the same observer (J. F. D.). A similar graph was obtained when four different observers sorted the same batch of drawings. 69 per cent. of the amoebae were classified correctly as 'typical P' and 'typical D' respectively, and 25 per cent. were placed in each of the categories 'like P' and 'like D'. For each group some amoebae were classified outside the species: 6 per cent. of *A. proteus* drawings were classified as 'like D' and 6 per cent. of *A. discoides* drawings as 'typical P'. Thus it seems that, although the method of shape-sorting gives a clear-cut separation of the two species when cultures are examined, there are always some individual amoebae which are atypical in this respect.

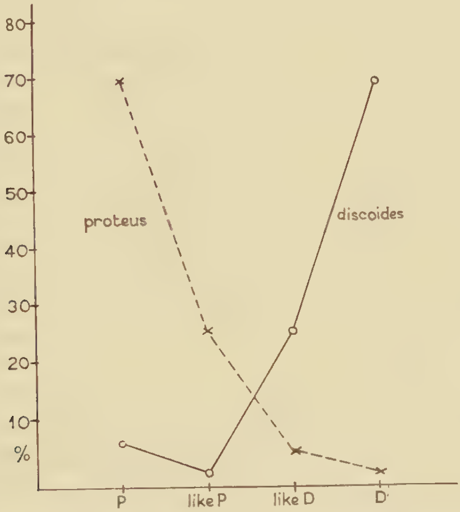

FIG. 2. Graphical presentation of the results of repeated sorting of 35 outline drawings. The percentage of drawings placed in each category is plotted. For explanation see text.

Schaeffer (1916) states that the mode of formation of pseudopods is different in *A. proteus* and *A. discoides*, and that 'the most distinctive morphological characteristic of *A. proteus* is the possession of longitudinal grooves on the surface when in locomotion'. These grooves are said to be 'never absent except in such individuals as have not divided for many days'. Whereas we agree that such 'grooves' are usually (though not always) seen in active specimens of *A. proteus*, we cannot agree that they are never present in *A. discoides*. They therefore cannot be used as an absolute distinguishing mark of *A. proteus*. The same may be said of the other distinguishing features in the cytoplasm of the two species as described by Schaeffer, e.g. shape, size, and number of crystals present, number of refractile 'nutrition' spheres, speed of streaming, and colour of the cytoplasm by transmitted light. None of these features was found to be of decisive diagnostic value for the particular cultures used in this study. It may be noted at this point that descendants of a culture of *A. proteus* obtained from Dr. J. A. Dawson of New York in 1950 correspond much more closely to Schaeffer's description of this species than does the Glasgow stock.

A very noticeable difference between the two species is the *size* of the animals: specimens of *A. proteus* are in general about one-third longer than *A. discoides*. No volume measurements were made. Size differences may be useful in distinguishing between cultures of the two species but cannot be applied to individuals, as there is considerable variation.

It was thought that dividing amoebae of the two species might display some characteristic difference and therefore observations and camera lucida sketches of a considerable number of dividing amoebae were made (Doljanski, 1954). The mode of cytoplasmic division in both species was found to be as described by Dawson and others (1937) and Chalkley and Daniel (1933) for *A. proteus*. Again no decisive difference between the two species was found.

Thus it is seen that no means of distinguishing between a single *Amoeba proteus* and a single *A. discoides* was found from an examination of the locomotion of the animal or the appearance of the cytoplasm, but flourishing *cultures* could be distinguished readily by sorting outline drawings of random samples.

The possibility of finding characteristic differences between the *nuclei* of the two species was then investigated. In both species the nucleus rolls about freely in the internal cytoplasm and is habitually found in the posterior third of the animal. It is prevented from being carried forward in the stream by the network of fine cytoplasmic strands which exists in the anterior part of the amoeba but which is continually being dissolved at the tail end (Goldacre, 1952). The shape of the nucleus of *A. proteus* was found to be basically discoid, but nuclei are often folded or notched so that they can take on various shapes. In young specimens (up to about 6 hours after division) the nucleus is discoid and has a smooth outline. It is easily deformed temporarily by the cytoplasmic streaming. In very old amoebae the nucleus is folded into bizarre shapes.

The nucleus of *A. discoides* is discoid and is neither folded nor notched except in very old or starved specimens. Our observations on the interphase nuclei of the two species agree with those of Schaeffer (1916) and of Hayes on *A. discoides* (1938). The fact which emerges from a study of the shapes of nuclei is that if a young active amoeba has a notched or folded nucleus, then it is almost certainly *A. proteus*. But an old amoeba with a folded nucleus, or any amoeba with a smooth discoid nucleus, cannot be readily identified in this way.

The size of the nuclei, like the volume of the amoeba, is greater in *A. proteus* than in *A. discoides*. Schaeffer (1916) gives the average diameters of the nuclei of two species as 46 by 15 μ for proteus and 40 by 18 μ for discoides. He does not state on how many measurements these averages were based, nor the degree of variation.

Since the nucleus increases its volume between two divisions, it is necessary to measure a number of nuclei in order to get an average value for a particular culture. Measurements of the largest diameter of the nuclei of random samples taken from cultures of the two species were made, and the distribution of diameters plotted. Only mononucleate amoebae were used. The experiments were designed to find out whether the distribution of diameter and the average nuclear diameter are constant for particular clones under different conditions, or vary with the age, state of nutrition, &c. of the culture; whether the average nuclear diameter reflects the size of the parent nucleus; and whether there is a significant difference between cultures of *A. proteus* and *A. discoides*.

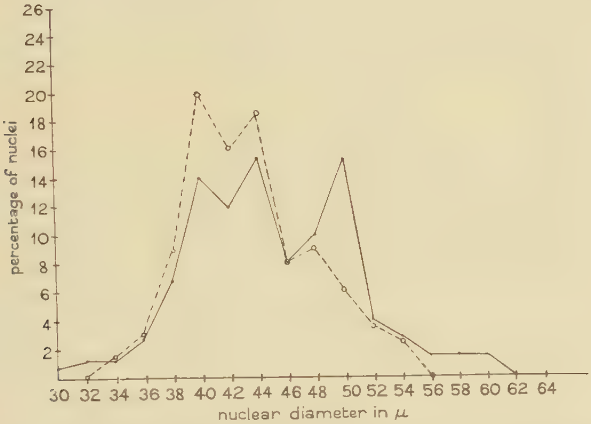

FIG. 3. Nuclear diameters of amoebae in mass cultures compared with those in rapidly growing cultures.

Solid line: *A. proteus* in mass cultures (cultures P2–5, table 2).
Broken line: *A. proteus* in rapidly growing cultures (serial numbers P13–15, table 1).

Tables 1–4 (see end of paper) and figs. 3 and 4 illustrate the results of this study. The cultures are grouped as 'rapidly growing', i.e. 'young' clones (tables 1 and 3) and 'mass cultures' (tables 2 and 4). The 'young' clones were set up from a single amoeba not more than 3 weeks before the measurement of the nuclei and contain between 50 and 120 amoebae. The 'mass cultures' contain over 1,000 amoebae in a 10 in. culture dish. It is seen that there is no great difference between the average nuclear diameters of recently established and older clones. The former tend to contain more amoebae with small nuclei: the rate of division is often higher in 'young' clones. Fig. 3 illustrates this difference for *A. proteus*: the graph for the rapidly growing 'young' clones is somewhat displaced to the left. In *A. discoides* the displacement of the curve for 'young' clones was less marked. Thus age of a culture by itself has no great influence on the distribution of nuclear diameter nor on the average, and we felt justified in representing all the mass cultures together (fig. 4) in order to show the average pattern of distribution of nuclear diameters in *A. proteus* and *A. discoides*.

Conditions of culture, rather than age, might affect nuclear diameters; hence well-fed and active as well as starved cultures were chosen for measurement. Again no marked constant difference was detected. For example, in table 2, P3 and P4 represent an underfed and a well-fed mass culture respectively: the average nuclear diameters are the same. In table 4, culture D5*c* i a starving culture and its average nuclear diameter, 38 μ, is intermediat between those of the two active, well-fed cultures, D15 (36 μ) and D16 (40 μ).

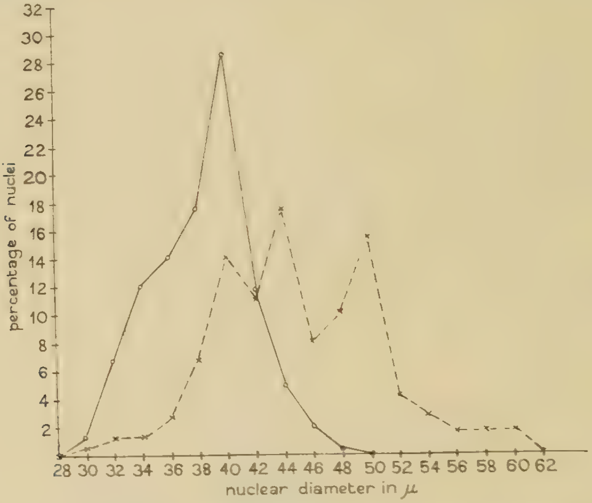

FIG. 4. Nuclear diameters of amoebae in mass cultures.
Solid line: *A. discoides* (cultures D5*c*, 15 and 16, table 4).
Broken line: *A. proteus* (cultures P2–5, table 2).

Yet it should not be concluded that external conditions have no effect on the nuclear diameters, otherwise it would be difficult to explain why the same culture examined at different times gives slightly different average values, e.g. table 3, D5 *a* and *b* and table 4, D5*c*, and why different cultures kept under as nearly as possible identical conditions give almost identical average values. For example, table 3, D10, 11, and 12 all have a 37 μ average; however, of the cultures P13, 14, and 15 on table 1, which are also under 'equalized' conditions, only two show identical average diameters. These 6 cultures were 'equalized' as follows: 10 active amoebae were picked out from each of 6 different cultures and placed in solid watch-glasses containing dense 'food' culture. After 8 days the resulting cultures were used for measurements. P20 (table 1) and D19 (table 3) were also treated in this way but not at the same time as the other six cultures.

We believe we have shown that the size of the nucleus of the parent of a clone does not influence the average nuclear diameter of the resulting culture. Four cultures of *A. proteus* (table 1) and 4 cultures of *A. discoides* (table 3) illustrate this point.

Also the operation of nuclear transfer between amoebae of the same species does not appear to influence the average nuclear diameter of the resulting clone (table 1, P10 and 11*a*; table 3, D2*a*, 8*a*, 13, 14, 17, 18) nor the distribution of nuclear diameters in a culture: such cultures may be considered normal in every respect (see Lorch and Danielli, 1953, for methods used in nuclear transfer).

The difference between the nuclear diameters of *A. proteus* and *A. discoides* is illustrated in fig. 4, which shows the nuclear diameters of 250 specimens of *A. discoides* and 340 of *A. proteus*, both from mass cultures under various conditions. It is seen that the shapes of the curves are markedly different, but there is considerable overlap. The average nuclear diameters for these amoebae were 38·2 (s.d. 3·6) μ for *A. discoides* and 45 (s.d. 5·6) μ for *A. proteus*.

DISCUSSION

The following main points emerge from a review of the results obtained.

1. Observation of the mode of pseudopod formation and of the cytoplasmic inclusions did not reveal any constant difference between *A. proteus* and *A. discoides*. Specimens of the former species are usually larger, but there is much variation in size in both species.

2. The pattern of locomotion as demonstrated by successive outline drawings was found to be typical for each species, but some atypical amoebae were found in each culture tested.

3. The nucleus of *A. proteus* is generally larger than that of *A. discoides* and is often notched or folded, whereas that of *A. discoides* is usually discoid with a smooth outline. Again there are exceptional specimens in respect of each of these features.

It is perhaps not remarkable that no clear-cut differences between individuals of *A. proteus* and *A. discoides* were found under the present conditions. The extent of biological variation among protozoa of the same species and even within a clone is always considerable. The variation is much reduced when the animals are cultured under constant conditions, preferably in a sterile medium as has been found with various ciliates. Some soil amoebae (Singh, 1950) have been cultured under relatively constant conditions on solid media. Singh's method of using a single strain of bacteria as food-supply for amoebae on non-nutrient agar greatly reduces the variability within the clones of soil amoebae used. Unfortunately the large free-living amoebae of the *proteus* group are not bacterial feeders, nor have they ever been cultured in the absence of living food animals. Hence, without a controllable food supply, the conditions for a comparison of the two species are necessarily imperfect. Nevertheless we have shown that certain characters are species-characteristic, namely the shape of the active animal and the distribution of nuclear diameters within a clone.

An analysis of the graphs of nuclear diameters of the cultures set out in tables 1–4 shows that there tend to be 3 maxima or well defined 'shoulders' in the *A. proteus* graphs (at 40 μ, 44 μ, and 50 μ), and two maxima in the

A. discoides graphs (at 34 μ and 40 μ). See, for example, fig. 4. *A. proteus* graphs were never found to have a 'peak' below 38 μ, whereas in the *A. discoides* graphs the 'peak' at 50 μ was absent. This is illustrated by table 5.

We are not able to express an opinion on the significance of the maxima in the graphs of nuclear diameter. Under the relatively constant culture conditions we used, the maxima were constantly found. Fig. 5 shows two sets of measurements made on the same culture with an interval of two years between the two sets. These two sets are closely similar. Fig. 3 shows that even when cultures are compared in widely different states of nutrition, displacements of the maxima are not very striking.

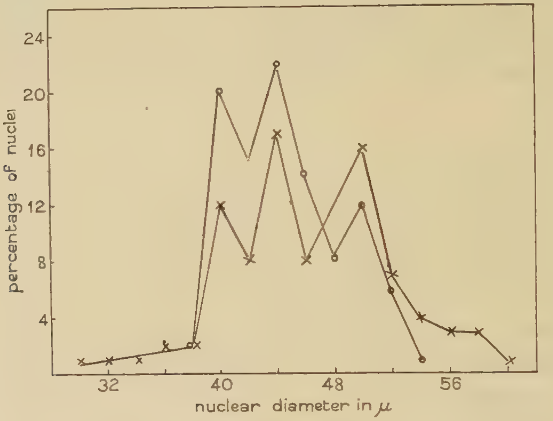

FIG. 5. Nuclear diameters in a mass culture of a clone of *A. proteus*. There was an interval of 2 years between the two sets of measurements. Note that the maxima are at the same diameters (40, 44, and 50 μ) in both cases. O, 1949; X, 1951.

Although *individual* amoebae cannot necessarily be classified by their nuclear diameter, the average nuclear diameter of a random sample of at least 50 amoebae from a healthy culture usually enables one to identify the culture, as may be seen by comparing tables 1 and 2 with tables 3 and 4.

Differences in the mode of binary fission sometimes provide diagnostic features for the classification of protozoa. For instance, Singh (1951) revised the classification of some soil amoebae according to type of nuclear division. It has been mentioned that no difference in the mode of *cytoplasmic* division of *A. proteus* and *A. discoides* was found by Doljanski (1954). An examination of stained preparations of division-stages of the two species also yielded negative results: the mitotic figures of the two species were indistinguishable. Doljanski was unable to confirm the differences in the shape of the spindle described by Hayes (1946).

Certain species of amoebae form typical cysts which aid in their identification (Singh, 1951). The question of the encystment of the large fresh-water amoebae has recently been brought up again by Galbraith and Taylor (1950), who claim to have demonstrated that '*Amoeba proteus* in the young encysted stage can survive desiccation and is thus capable of dispersal by wind. . . .' The authors do not, however, present any conclusive evidence of the existence of such encysted stages, either in this or in earlier publications (Taylor, 1924, 1927) and we fully agree with Johnson (1930) and Halsey (1936), who, after a critical study of the alleged life cycle of *A. proteus* and *A. dubia*, concluded that these amoebae reproduce only by binary fission. The same seems to apply to *A. discoides* and we were unable to confirm Hayes's (1938) observations of agamontogony in this species. Hence the life cycles of *A. proteus* and *A. discoides* do not provide any features which might serve to differentiate between the two species.

So far only morphological differences between the species have been studied. It is not unlikely that significant differences may be found in their physiological or serological properties. That this may be so is suggested for instance by the work of Dawson and Belkin (1928, 1929), who studied the digestion of oils by *A. proteus* and *A. dubia*. They found significant differences between the species, both in their ability to digest certain oils and in the nature of the pellicle. Thus under certain conditions *A. dubia* undergoes the phenomenon of 'capping' with oil, but no permanent capping was found to take place with *A. proteus*. (In some preliminary experiments with *A. discoides* it was found that it behaves like *A. proteus* in this respect, i.e. oil droplets did not form permanent 'caps'.)

Physiological differences between species of amoebae were also found by Andresen and Holter (1949), who examined the proteolytic enzyme contents of *A. proteus*, *Chaos chaos* (Schaeffer), and *Pelomyxa palustris* (Greef). Work on possible physiological and serological differences between *A. proteus* and *A. discoides* is still in progress.

However, the only differences which we have found between the two species which at present permit of quantitative study are the form assumed in moving and the distribution of nuclear diameters in a culture. These two factors were studied after making nuclear transfers between species. The results will be discussed in a subsequent paper.

ACKNOWLEDGEMENTS

The preliminary stages of this work were financed by a grant from the British Empire Cancer Campaign, and the later stages by a grant from the Nuffield Foundation. We are indebted to the Rockefeller Foundation for a gift of microscopes, to Professor A. Haddow of the Chester Beatty Research Institute for loan of apparatus, and to the Royal Society for the loan of micromanipulators.

TABLE 1. Amoeba proteus. *Nuclear diameters of rapidly growing cultures. Th* *standard deviations of the average nuclear diameters are approximately* 5·5.

Serial number	*Approx. number of amoebae present*	*Age of clone (days)*	*Date examined*	*Number measured*	*Average nuclear diam. μ*	*Nuclear diam. of parent of clone*	*Comments*
P1	60	16	15/12/50	47	43	..	..
P5*a*	50	20	2/3/51	45	47	..	cf. P5*b*, table 2
P7	100	15	10/2/51	100	44	..	..
P16	90	16	30/4/51	50	47	50–58*	*measured on different days
P17	100	16	30/4/51	50	45	60	..
P18	>100	17	1/5/51	50	45	44	..
P19	>100	18	2/5/51	50	45	42	..
P13	100	†	28/4/51	50	42	..	†P13–15 and P20 kept under 'equalized' conditions. See text. Cf. D10–12 and D19, table 3.
P14	70	..	28/4/51	50	42	..	
P15	50	..	28/4/51	38	44	..	
P20	100	..	22/11/51	50	43	..	
P10	80	20	16/4/51	50	44	52	clones derived from parent whose nucleus was replaced by that of another *A. proteus* (homotransfer)
P11*a*	50	14	10/4/51	50	41	48–68	

TABLE 2. Amoeba proteus. *Nuclear diameters of mass cultures (clones) of over* 1,000 *amoebae in different conditions. The standard deviations of the average nuclear diameters are approximately* 5·5.

Serial number	*State of culture*	*Date examined*	*Number measured*	*Average nuclear diam. μ*	*Comments*
P2*a*	some active, some floating	8/2/51	47	43	same culture on different days
P2*b*	active	20/3/51	100	44	
P3	not numerous, underfed	5/3/51	44	45	..
P4	well fed, active	3/3/51	100	45	..
P5*b*	10 weeks old, mostly active	23/4/51	50	42	same culture as P5*a* (table 1) 7 weeks later
P11*b*	3 weeks old, large stellate amoebae, few food vacuoles	23/4/51	50	46	Homotransfer, same culture as P11*a* (table 1) 13 days later

TABLE 3. Amoeba discoides. *Nuclear diameters of rapidly growing cultures. The standard deviations of the average nuclear diameters are approximately* 3·5.

Serial number	*Approx. Number of amoebae present*	*Age of clone (days)*	*Date examined*	*Number measured*	*Average nuclear diam. μ*	*Nuclear diam. of parent of clone*	*Comments*
D1	80	16	15/12/50	47	35	33	..
D5*a*	80	15	1/3/51	50	40	..	same culture. Cf. D5*c*, table 4
D5*b*	200	20	6/3/51	100	39	..	
D10	200	*	28/4/51	50	37	..	*D10–12 and D19 kept under 'equalized' conditions. See text. Cf. P13–15 and P19
D11	120	..	28/4/51	50	37	..	
D12	120	..	28/4/51	50	37	..	
D19	100	..	22/11/51	50	40	..	
D2*a*	60	12	1/2/51	47	36	..	*clones derived from homotransfers.* Cf. table 4
D8*a*	70	14	10/4/51	50	34	..	
D13	100	16	26/4/51	50	39	30	..
D14	100	16	26/4/51	50	39	36	..
D17	80	18	28/4/51	50	39	30	..
D18	80	18	28/4/51	50	42	42	large stellate amoebae probably no divisions for 2 days

TABLE 4. Amoeba discoides. *Nuclear diameters of mass cultures* (*clones*) *of over* 1,000 *amoebae in different conditions. The standard deviations of the average nuclear diameters are approximately* 3·5.

Serial number	*State of culture*	*Date examined*	*Number measured*	*Average nuclear diam. μ*	*Comments*
D5*c*	starving, sluggish .	27/3/51	50	38	cf. D5*a* and *b* (same culture) table 3
D15	small stellate amoebae, flagellate food only .	8/3/51	100	36	..
D16	very active, well fed .	13/3/51	100	40	..
D2*b*	..	23/3/51	50	39	*derived from homotransfers.* same culture as D2*a* (table 3) 8 weeks later.
D2*c*	..	29/3/51	50	36	
D8*b*	..	23/4/51	50	38	same culture as D8*a* (table 3) 2 weeks later

TABLE 5. *Table indicating the nuclear diameters at which peaks occur on histograms. See text.* * *indicates greatest maximum*

Culture	*Nuclear Diameter μ*										
	32	34	36	38	40	42	44	46	48	50...	...58
A. proteus											
(*a*) rapidly growing											
1. young clones . . .					o		o			o	
2. 'equalized' conditions . .					o		o			o	
3. young clones from homotransfers				o	o		o			o	
(*b*) mass cultures											
1. not operated . . .					o		o*			o	
2. from homotransfer (one culture)							o*			o	o
A. discoides											
(*a*) rapidly growing											
1. young clones . . .		+		+							
2. 'equalized' conditions . .		+			+						
3. young clones from homotransfers		+			+						
(*b*) mass cultures											
1. not operated . . .		+			+*						
2. from homotransfers . .	+				+*						

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Nuclear Transplantation in Amoebae. II

The immediate results of transfer of nuclei between *Amoeba proteus* and *Amoeba discoides*

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SUMMARY

1. A description is given of a method for replacing the original nucleus of an amoeba by a nucleus from another amoeba. This method has been used to study transfers of nuclei within a species (homotransfers) and between species (heterotransfers). The species used were *A. proteus* and *A. discoides*.

2. Removal of the nucleus from an amoeba inactivates the animal, so that movement is sporadic and unco-ordinated, digestion ineffective, and death results after 10–20 days.

3. Renucleation results in reactivation with both homo- and heterotransfers. Reactivated homotransfers normally are able to divide and form mass cultures. Heterotransfers are less often able to divide and very seldom form mass cultures.

4. Amoebae taken at random from a mass culture divide after an interval which appears to be composed of a lag t_l in which the processes leading to division are inactive, followed by an interdivision period t_d during which preparation for division is active. Experiments are given showing the effect of nuclear transfer on t_l and t_d.

5. After heterotransfer nuclei of either species may grow and divide in foreign cytoplasm. Before the first division after heterotransfer a cytoplasmic effect on nuclear size is observable, and after the first division the cytoplasm has a dominant effect in determining nuclear diameter.

6. When curves are plotted of the frequency of occurrence of nuclei of various diameters in pure cultures, maxima are found in the curves characteristic of the species. After nuclear transfer the cytoplasm and nucleus both have a strong influence in determining the position of the maxima.

7. Studies of the form assumed by heterotransfers show that, although there is a measurable influence of the nucleus on the form soon after transfer, this influence disappears almost entirely in 6–12 days, after which the determination of form is almost entirely cytoplasmic.

8. The results given under points 4, 5, 6, and 7 are based on the study of animals immediately after transfer and for the subsequent two divisions only. Longer-term effects will be discussed in another paper.

INTRODUCTION

IN a preliminary communication (Lorch and Danielli, 1950) we have pointed out that the simplest way to obtain unequivocal evidence of the extent to which irreversible changes occur in nuclei and cytoplasm during differentiation would be to transplant nuclei and cytoplasm from one type of cell to another. In the present work the technique of nuclear transplantation in amoebae, initiated by Commandon and de Fonbrune (1938, 1939), has

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been used to investigate the extent to which species differences are maintained by the nucleus and cytoplasm respectively. The two species used were *Amoeba proteus* and *Amoeba discoides*. Their species characters were discussed in an earlier paper (Lorch and Danielli, 1953). The two characters studied are (*a*) nuclear size and (*b*) the pattern of locomotion. The extent to which these characters are modified by substituting a foreign nucleus in place of the original nucleus is discussed.

This work incidentally affords opportunities for observations on enucleated amoebae and for studying the possible functions of the nucleus in these animals.

MATERIAL AND METHODS

Stocks of *A. proteus* and *A. discoides* were kindly supplied by Sister Monica Taylor and Sister Carmela Hayes. The technique of establishing clones has been described (Lorch and Danielli, 1953): the same technique was also used for raising clones from operated amoebae. All experiments were done on animals from clone cultures.

The methods of enucleation and nuclear transfer were essentially those of Commandon and de Fonbrune (1938, 1939). The amoebae to be operated upon were distributed in shallow hanging drops of culture medium (Chalkley, 1930) in a paraffin chamber (Commandon and de Fonbrune, 1938). For a successful operation the angle of contact of the aqueous drop must be neither too big (convex drops on a greasy surface) nor too small (spreading drops on a freshly cleaned surface). To ensure this, the coverslips, which were stored in strong chromic acid, were washed in running water for 1–2 minutes, rinsed in distilled water, and left to dry standing vertically under a cover for 12–24 hours. Amoebae were found to be less liable to cytolyse during an operation if a trace of protein was present in the drop. Optimum conditions were obtained by cytolysing one amoeba in each drop before adding the experimental animals.

The micro-instruments were made on the de Fonbrune micro-forge, and were used in conjunction with a de Fonbrune micro-manipulator. A glass hook (about 250 μ in internal diameter) was used to hold the amoebae against the meniscus of the drop. Amoebae were enucleated by pushing the nucleus out with a blunt micro-needle. A small loss of cytoplasm also occurs. A nucleus may be transferred from one amoeba to another by the same technique: the donor's nucleus is pushed through the cell membranes into the host's cytoplasm, both amoebae being held tightly in a hook. Immediately after the operation the amoebae were released from the hook and kept under observation in the drop for up to half an hour. They were then transferred by means of a mouth pipette to individual solid watch glasses containing ciliates and flagellates. Subsequent observations were made either in the solid watch glasses or by placing the amoebae in hanging drops in the paraffin chamber. The latter method was used when it was desired to measure the nuclear diameter by means of an ocular scale, or to make camera lucida drawings.

Outline drawings of active amoebae were made at 1 minute intervals for 4–6 minutes at various times after nuclear transfer.

Where a nucleus was transferred to the cytoplasm of another individual of the same species we term the operation a homotransfer, whereas a transfer of a nucleus from one species to another is termed a heterotransfer. For convenience in reference the following symbols have been used: D and P indicate *Amoeba discoides* and *Amoeba proteus* respectively, and the suffixes *n* and *c* indicate nucleus and cytoplasm respectively. Thus $P_n \rightarrow D_c$ represents a heterotransfer in which a *proteus* nucleus was transferred to *discoides* cytoplasm, and an individual $P_n 2D_n D_c$ indicates an amoeba consisting of *discoides* cytoplasm containing one *proteus* nucleus and two *discoides* nuclei.

RESULTS

Enucleation of amoebae

When the nucleus was pushed out of an amoeba with a minimum of disturbance, the animal resumed normal movement as soon as it was released from the hook. After about 30 seconds the movements began to be less co-ordinated and changes of direction became more frequent. The pseudopods tended to be short and blunt. The animal gradually lost contact with the coverslip and tended to drift to the centre of the drop, instead of crawling round its outer edge. Within 10 minutes of operation, all enucleated amoebae assumed an appearance typical for this stage; they were spheroidal, unattached to any surface, and covered with short blunt pseudopods which were slowly pushed out and withdrawn again, giving the animal's surface a corrugated appearance. Such amoebae appear dark by transmitted light and somewhat resemble division spheres. After about 24 hours in a dish the enucleated amoebae displayed intermittent periods of apparently normal amoeboid movement. But most of the time between the first and seventh day after enucleation the animals were found floating freely in the dish. The slow streaming of an enucleated amoeba did not result in displacement of the animal as a whole. During the periods of normal activity enucleated amoebae were seen to engulf food organisms, but they seemed to be unable to digest or even kill them. Enucleated amoebae were seen with active ciliates trapped in food vacuoles for days, whereas a normal amoeba usually kills its prey within a few minutes. When an enucleated amoeba was placed in a shallow hanging drop and prodded with a micro-needle it could be induced to crawl on the coverslip, but co-ordinated movements ceased again after about 10 minutes. The functioning of the contractile vacuole seemed to be unimpaired. After 6–8 days the bouts of normal movement ceased and the amoebae, which assumed a spheroidal, sausage, or stellate shape, remained quiescent at the bottom of the dish. They responded very little to light or prodding at this stage. The viscosity of the cytoplasm was decreased: Brownian movement of the small cytoplasmic inclusions became evident and the crystals and spheres sank to the lower surface of the amoeba. The animals were easily deformed by pressure with a micro-instrument and had a tendency to cytolyse when put on a coverslip.

Most enucleated amoebae died within 15 days, though a few lived as long as 20 days (see also Ord and Danielli, 1953). *Amoeba discoides* tended to die somewhat sooner than *A. proteus*. The removal of one nucleus from a binucleate amoeba was followed neither by abnormal behaviour nor by death.

Nuclear transplantation

General effects. When a nucleus of the same or of a foreign species was successfully inserted into an enucleated amoeba immediately after the enucleation, normal streaming continued indefinitely, i.e. the 'corrugated' state did not set in. If the enucleated amoeba was already rounded up and unattached, say half an hour after enucleation, and a new nucleus was inserted at that stage, then a dramatic 'reactivation' took place; the amoeba stretched out, became attached to the coverslip, and started streaming in a co-ordinated way. The transferred nucleus was immediately drawn into the normal position in the cytoplasm in the posterior third of the animal. Sometimes this 'reactivation' took place within a few seconds of the transfer, in other cases the amoeba became active after a latent period of several hours, depending on the mechanical damage done during the operation. Reactivation still occurred when a nucleus was implanted 3 days after enucleation.

As de Fonbrune has pointed out, a new nucleus is only 'accepted' by an amoeba if it is pushed directly from the donor's to the host's cytoplasm, i.e. without touching the aqueous medium. Other attempts at transplantation failed because this was not realized (Clark, 1943). In our experiments, when a nucleus came into contact with the surrounding fluid it was immediately and irreversibly damaged. Such a nucleus was treated by the host as a foreign body: instead of being drawn into the central stream of cytoplasm to take up the normal position of a nucleus it became situated in the tail end of the animal whence food vacuoles are normally discharged, and was eventually extruded.

A mechanically damaged nucleus or a nucleus covered by the cell membrane of the donor was treated similarly. The latter case arose if, during a transfer, the host's or doner's membrane did not break at the moment the nucleus was pushed into the host, so that a loop of membrane was pushed into the host's cytoplasm together with the nucleus. Portions of the outer membrane of an amoeba were never tolerated in the interior of the animal, even if the membrane came from the same animal. A similar observation was made by Okada (1930), who attempted to push fragments of *Pelomyxa* into an intact animal but found they were invariably extruded. Fusion occurred only after the 'pellicle' had been stripped from the parts to be joined. In *Arcella*, on the other hand, fusion between cytoplasmic fragments and the main body have been reported (Reynold, 1924).

The proportion of reactivated animals, divided animals, and clones. Detailed records have not been kept of the majority of nuclear transfers carried out. The figures given in table 1 (see end of paper) are for comparable sets of transfers of the four types $P_n \rightarrow P_c$, $D_n \rightarrow D_c$, $P_n \rightarrow D_c$, and $D_n \rightarrow P_c$. Each individual was carefully examined either until death intervened or to the stage at which a culture of over a hundred individual amoebae had been obtained. The data presented in the table show that with homotransfers about 90 per cent. are successfully reactivated, about 75 per cent. divide at least once after the transfer, and that about 70 per cent. go on to give successful cultures. Reactivation is almost equally successful with heterotransfers, but division is much less frequent, and of the heterotransfers reported in the table, none gave mass cultures.

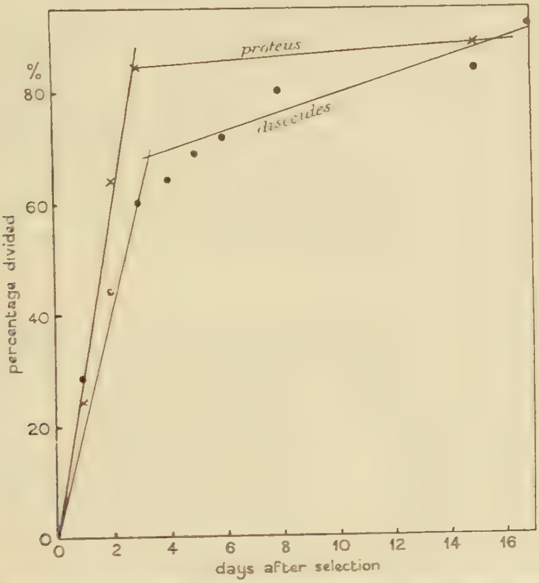

FIG. 1. The interval between selection and division for individual amoebae selected at random from mass cultures and then kept in individual watch-glasses.

Duration of active life and time of cytolysis of transfers which failed to divide. Although the majority of enucleated amoebae are reactivated by renucleation, not all reactivated animals divide. In such animals it is easy to determine the duration of active life and the onset of cytolysis. Information on these points is set out in table 2. In respect of duration of active life, P_nP_c, D_nD_c, and D_nP_c appear to be favourable combinations, whereas P_nD_c is relatively unfavourable. So far as onset of cytolysis is concerned, behaviour in this group of animals is cytoplasmically determined, P_c being more resistant to cytolysis than D_c. It is a striking fact that although renucleation may restore activity, unless the renucleation is also sufficiently successful to permit division, cytolysis occurs at about the same time as in amoebae left enucleated.

The delay in division caused by nuclear transfer. Fig. 1 shows the rate of division for normal amoebae and fig. 2 the rate of division for nuclear transfers for the first division after operation. The operation delays division. In a mass culture, the population of which is homogeneous, there should be a typical time t_d between divisions. If we take a number (n_0) of individuals at random from such a culture, all these individuals should have completed division after a further lapse of time t_d. If the n_o individuals taken from the mass culture are statistically representative of the culture, the proportion n/n_o which have divided at intermediate times t should lie on the straight line

$$n/n_o = t/t_d \tag{1}$$

As a result of an operation, or any other procedure, there may be a lag t_l before the processes leading to division begin again, and also t_d may change.

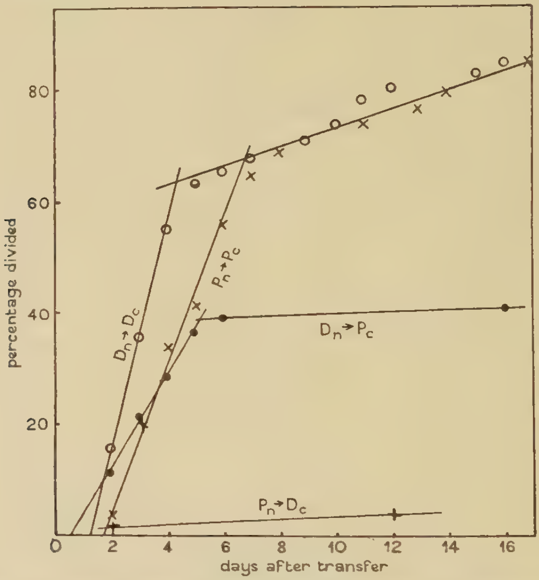

FIG. 2. The interval between nuclear transfer and division for homotransfers and heterotransfers.

In such cases, if the population remains homogeneous, division of a random selection should fall on a straight line

$$n/n_o = \frac{t-t_l}{t_d} \tag{2}$$

The points plotted in fig. 2 show that for any group of experiments the points fall approximately on either one or two straight lines. Where the points fall on one line all the individuals divide rapidly. Where the points fall on two lines there is also a group of slow dividers. From the data of figs. 1 and 2 values of t_d and t_l have been calculated (table 3). Individuals from mass cultures were observed by placing in watch glasses with ample food. For most individuals under such conditions, if unoperated, the lag t_l was negligible and the interval between divisions was about 3–4 days. A minority of individuals were slow dividers and behaved quite differently, showing a lag of several days and an interval between divisions of about 15 days. When animals were subjected to homotransfer, the majority of animals remained fast dividers, but exhibited a lag of 1–2 days and perhaps a small increase in t_d also. The homotransfers which were slow dividers showed the same characteristics as unoperated slow dividers, having t_l of a few days and t_d about 15 days. With heterotransfers the situation was sharply different. For $D_n \rightarrow P_c$ those animals which divided showed a small lag, and a t_d of about 5 days: there were no slow dividers. For $P_n \rightarrow D_c$ too few animals divided to permit analysis.

From these results it appears that animals in a culture are in either of two different physiological states. In one state $t_l \simeq 0$, and the interval between divisions is short, about four days. In the other state there is a lag of several days and the interval $t_d \simeq 15$ days. There are very few animals, if any, showing behaviour intermediate between these two states. So far as can be seen from these results, homotransfer makes little difference to these states, and operations with both slow and fast dividers are successful. But with heterotransfers only operations of the type $D_n \rightarrow P_c$ show a good proportion of successes, and these successes appear to be confined to fast dividers.

Delayed transfer

The great majority of the nuclear transfers reported in this paper were carried out a few minutes after enucleation. But renucleation may still be successful after several days in the enucleated condition. Table 4 shows experiments comparing the effect of immediate and delayed transfer. In general, delayed transfer is less successful than immediate transfer. Both with immediate and delayed transfers, homotransfers are more likely than heterotransfers to yield individuals capable of division.

Double transfers

Heterotransfers, although yielding many reactivated individuals and some individuals able to divide, give very few capable of giving mass cultures. It was thought possible that the failure to give mass cultures might be due to damage either to nucleus or to cytoplasm before sufficient mutual adaptation had occurred. It seemed possible that after a nucleus had been in a foreign cytoplasm for some time, it might with advantage be re-transferred to a fresh foreign cytoplasm, and then be replaced in the old foreign cytoplasm by a new nucleus. So far 10 experiments of this nature have been carried out, 8 with the transfer $P_n \rightarrow D_c$ and 2 with $D_n \rightarrow P_c$. The nuclei were re-transferred 24 hours after the original transfer into fresh foreign cytoplasm, and fresh nuclei then transferred into the original foreign cytoplasms. The re-transferred nuclei were able to activate the fresh foreign cytoplasms, and the fresh nuclei were able to activate the original foreign cytoplasms. But all the 20 individuals so obtained died without dividing.

Changes in nuclear diameter after transfer

In a previous paper it was shown that after cell division the diameter of the nucleus is at a minimum, and during growth of an amoeba the diameter increases to a maximum before division occurs again. With *A. proteus* th usual range of diameters is from about 36 μ to about 54 μ, and with *A. discoides* from about 30 μ to about 46 μ, although with both species exceptiona individuals may be found with diameters outside these ranges. The averag diameter, under our conditions of culture, is 45 μ for *A. proteus* and 38·2 μ

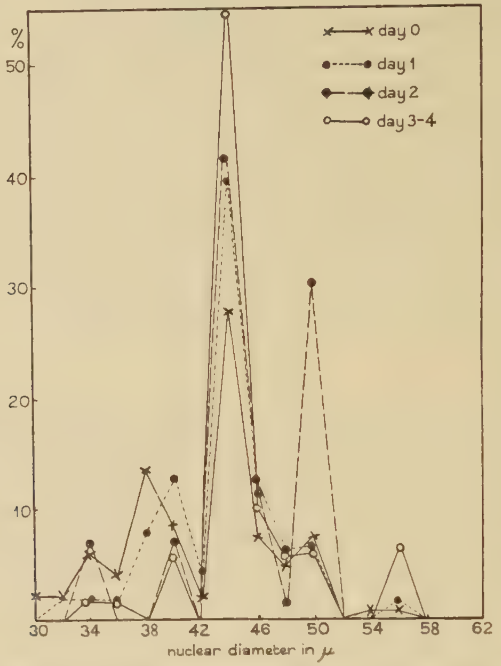

FIG. 3. The distribution of nuclear diameters for *proteus* nuclei in *discoides* cytoplasm, at various intervals after transfer.

for *A. discoides*. It was of interest to see whether, in heterotransfers, the behaviour of the nucleus would be determined by the nucleus or by the cytoplasm.

Figs. 3 and 4 show the distributions of nuclear diameters in the transfers $P_n \rightarrow D_c$ and $D_n \rightarrow P_c$ at various times after transfer, but before the first division after transfer. In the experiments recorded in these figures the nuclei to be transferred were taken at random from mass cultures, and the diameter of the nucleus measured a few minutes after the transfer was completed to obtain the points for day 0. It was necessary to measure the diameters *after* and not before transfer to avoid changes caused by differences in osmotic conditions in the two species. For P_nD_c the distribution of nuclear diameters at day 0 (fig. 3) was similar to that found with P_nP_c, with peaks at 38, 44, and 50 μ (see Lorch and Danielli, 1953). But by day 4 the distribution had changed markedly, with over 50 per cent. of the animals having a nuclear diameter close to 44 μ, the numbers of nuclei with other diameters being proportionately less. Table 5 shows the average diameters of the same set of ransfers: for P_nD_c there is probably no significant change in *average* diameter ver 4 days, although the *distribution* of diameters changes sharply. The explanation of this was found by plotting growth curves of individual nuclei as function of time: when the initial diameter of a nucleus in P_nD_c is greater han 44 μ, the diameter usually decreases with time; when the initial diameter s less than 44 μ, the diameter usually increases with time. As a result there is n accumulation of nuclei having a diameter of about 44 μ. The net result of these changes is to produce a curve of diameter distributions which in shape resembles that found for D_nD_c, but which has its peak at 44 μ instead of the value of 38 μ characteristic of D_nD_c. Thus in the period between transfer and division the distribution of nuclear diameters in P_nD_c is strongly influenced by both nucleus and cytoplasm.

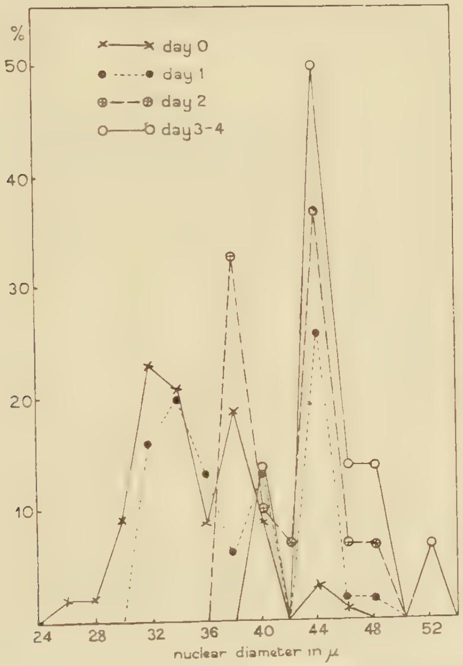

FIG. 4. The distribution of nuclear diameters for *discoides* nuclei in *proteus* cytoplasm, at various intervals after transfer.

With the transfers $D_n \rightarrow P_c$ the distribution of nuclear diameters (fig. 4) changes with time in a different manner. Almost all the nuclei grow, so that the distribution curve shifts along the diameter axis: by the fourth day the distribution of nuclear diameters is similar to that found with P_nP_c, except that there is a much greater proportion of nuclei of diameter 44 μ. As is shown in table 5, whereas with P_nD_c there was practically no increase in *average* nuclear size, with D_nP_c there was a rapid growth from 35 μ at day 0 to 50·5 μ at day 9. Fig. 5 shows the growth curves for 10 individual *discoides* nuclei in *proteus* cytoplasm, which were measured regularly for 7 or 8 days. Th growth rate was fairly regular, and as is indicated by table 6 the daily incre ments in the cube of the maximum diameter are fairly constant, suggestin that nuclear volume increases fairly steadily.

All the measurements of nuclear diameter mentioned so far were of nucle which had been transferred to foreign cytoplasm but had not yet divided Measurements of nuclear diameter of P_nD_c and of D_nP_c after the first division

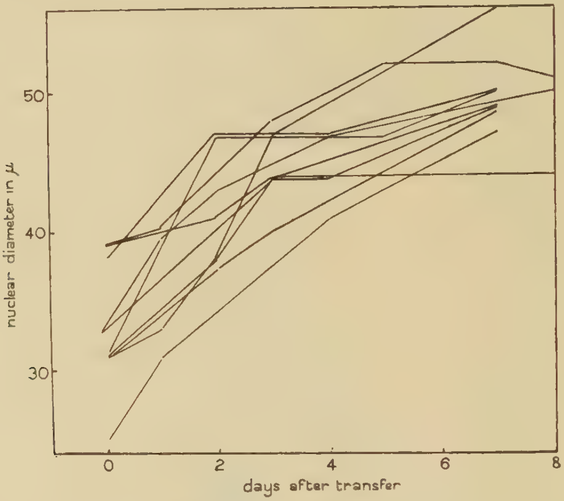

FIG. 5. The growth in diameter of ten *discoides* nuclei in *proteus* cytoplasm.

showed a still further degree of cytoplasmic control of nuclear diameter. The nuclei in both types of heterotransfer had a minimum volume after division, from which they grew to at least 44 μ, after which further cycles of division and growth occurred in some cases. The average of 19 measurements of nuclear diameter on D_nP_c after the first division was 44 μ, and the average of 12 measurements on P_nD_c was 38 μ. These average values are in each case typical of the cytoplasmic, and not of the nuclear species.

Table 7 shows a further group of measurements on heterotransfers before division. Animals in this group received a foreign nucleus which was replaced after 24 hours by a second foreign nucleus. The behaviour of the second nuclei was similar to that of the first nuclei. *Proteus* nuclei grew very little in *discoides* cytoplasm, whereas *discoides* nuclei grew rapidly in *proteus* cytoplasm, so that although the *discoides* nuclei were initially smaller than the *proteus* nuclei, after 24 hours the *discoides* nuclei were the larger. The rate of growth of the nuclei was largely determined by the cytoplasm.

The position of the maxima in nuclear diameter distribution curves

In the previous paper (Lorch and Danielli, 1953) it was shown that when curves were plotted of the frequency of occurrence of nuclear diameters in culture, maxima were displayed which were characteristic of a species. Curves for *A. proteus* and *A. discoides* both showed maxima at 40 μ, occasionlly replaced by maxima at 38 μ, but curves for *A. proteus* also showed maxima t 44 and 50 μ which were not shown by *A. discoides*. With *A. discoides* a maxinum was found at 34 μ which was not encountered with *A. proteus*. The greatest maxima are at 44 μ for *proteus* and 40 μ for *discoides*, which may shift to 40 μ and 38 μ respectively when the cultures are growing very rapidly. These results are summarized in table 8 together with a number of observations on heterotransfers: the data on heterotransfers are for individuals which had not divided between transfer and the recorded measurements of nuclear diameter.

For the heterotransfer $D_n \rightarrow P_c$ the positions of the maxima shift from being entirely in the *discoides* range soon after transfer (day 0) to being entirely in the *proteus* range by the fourth day after transfer. This change in position of the maxima is concomitant with the steady growth of *discoides* nuclei in *proteus* cytoplasm recorded in the previous section of this paper. For $P_n \rightarrow D_c$, maxima characteristic of both species are found even on the fourth day after transfer. Thus with P_nD_c the cytoplasmic effect is not so striking as with D_nP_c before division has occurred. On the other hand, as noted above, once division has occurred after heterotransfer the average nuclear size is almost entirely cytoplasmically determined for the F_1 and F_2 generations.

The effect of nuclear transfer on form during movement

In an earlier paper (Lorch and Danielli, 1953) it was shown that there was a characteristic difference between the forms of individuals of the two species when moving. There is considerable variation from individual to individual, so that any single amoeba cannot readily be ascribed to its species as a result of a single observation of its form. But if camera lucida drawings are made of the outlines of a number of amoebae, and sorted into typical *proteus* (P), like *proteus* (*l*P), like *discoides* (*l*D) and typical *discoides* (D), as described in the section on experimental methods, a striking statistical difference between the two species becomes apparent. As shown in fig. 6, of the individuals from a *proteus* clone about 70 per cent. are classified as P, another 15 per cent. as *l*P, and usually less than 10 per cent. as *l*D and D together. A corresponding relationship is found for a *discoides* clone.

Before it was possible to assess the effect of nuclear transfer on form of heterotransfers, it was necessary to assess the effect of homotransfer upon form. The results of such an investigation are recorded in figs. 6 and 7. In the former it is shown that the *immediate* effect of homotransfer is negligible: if there is any change, it is an accentuation of the normal difference between the two species. But fig. 7 shows that there is a significant alteration in the form of the F_1 and F_2 generations, particularly in *proteus*. This upset of the normal pattern disappears after the first few cleavages. But the existence of this upset means that, whereas before cleavage heterotransfers may be classified against either unoperated amoebae or undivided homotransfers, classification of the

F_1 and F_2 generations after heterotransfer must be made with reference to the F_1 and F_2 generations of homotransfers and cannot be made against any other standard.

Figs. 8 and 9 show the effect of heterotransfer on the form of amoebae after transfer but before division. Data are given for groups of amoebae studied at intervals of 1, 2–3, 4–6, and 7–12 days after transfer. In all groups the cytoplasmic character is the more evident. Soon after operation there is a marked displacement towards the nuclear species, but this rapidly diminishes, and after 6–12 days the influence of the nucleus has become negligible.

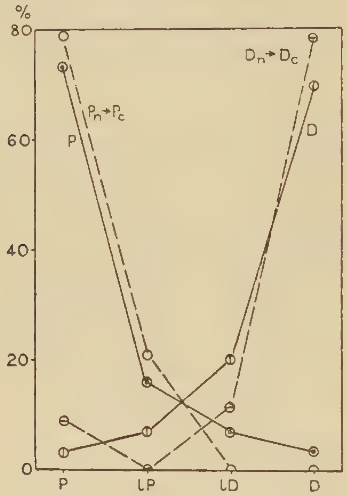

Fig. 6

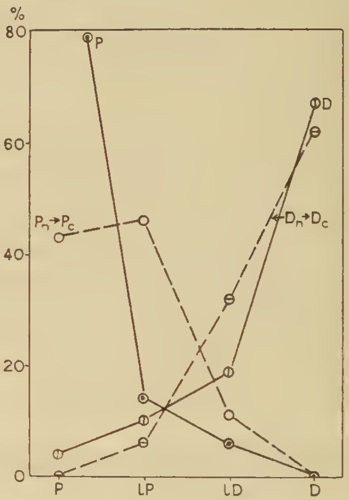

Fig. 7

Fig. 6. The distribution of types in a group of homotransfers (before division) as indicated by form when in movement. P = typical *proteus*; *l*P = more like *proteus* than *discoides*; *l*D = more like *discoides* than *proteus*; D = typical *discoides*. Curves for groups of unoperated amoebae are included for comparison.

Fig. 7. As fig. 6, but for the amoebae which have divided after homotransfer.

Fig. 10 shows similar data for the F_1 and F_2 generations after heterotransfer for D_nP_c. Insufficient records have been obtained for P_nD_c to justify analysis. It is evident from the figure that neither the F_1 nor the F_2 generation of D_nP_c shows a significant influence of the nucleus. Indeed, D_nP_c shows less disturbance from the typical *proteus* distribution than do the F_1 and F_2 generations of homotransfers.

Fig. 11 shows the effect of double nuclear transfer on the form of animals in movement, for $P_n \to D_c$. The original *discoides* nuclei were replaced by *proteus* nuclei which were left in the cytoplasm for one day, and then replaced by a second *proteus* nucleus. After the second transfer the influence of the nucleus is prominent. Comparison with fig. 9 shows that the nuclear influence is more pronounced than with single transfers: indeed, the nuclear influence is greater than in any transfer so far reported.

The conclusion to be drawn from the study of the shape of heterotransfers that form during movement is almost entirely determined by the species of e cytoplasm, at least up to and including the F_2 generation after transfer. he influence of the nuclear species is detectable soon after transfer, but tends diminish to negligible proportions during the course of 6–12 days.

DISCUSSION

Nuclear transfer with *A. proteus* and *A. discoides* is not a difficult technique or those with some aptitude for micro-dissection, and a reasonable degree of proficiency may be acquired by 3 months' practice. Success in transfer, however, is dependent upon the mechanical properties of the nucleus and of the cell membranes, and the application of this technique to other cells will be dependent upon the occurrence of favourable combinations of circumstances. Thus we have encountered great difficulty in making transfers between cells in developing sea-urchin eggs, and also between several species of small soil amoebae. Even with *A. proteus* and *A. discoides* the operation becomes impracticable if the animals are cultured above 20° C. Above this temperature the cell membranes become very elastic, and when an attempt is made to push a nucleus from one cytoplasm to another the membranes stretch and do not permit passage of a naked nucleus. If renucleation is to be effective, the nucleus must come into direct contact with the cytoplasm. If, after transfer, the nucleus is still surrounded by cell membrane it is treated as a foreign body, does not reactivate the cytoplasm, and is eventually ejected like a spent food vacuole. Similarly if a whole amoeba with its nucleus is inserted into enucleated

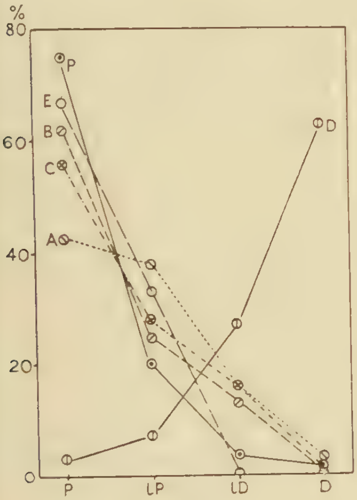

FIG. 8

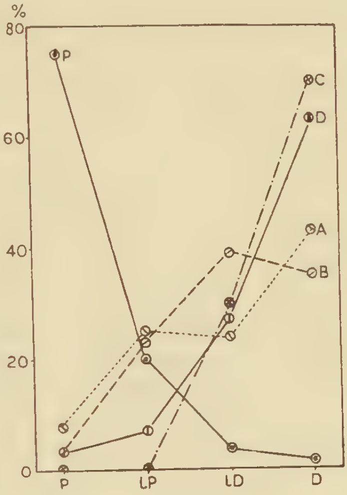

FIG. 9

FIG. 8. As fig. 6. Distribution of types in a group of the heterotransfer D_nP_c (before division). A = day 1; B = day 2–3; C = day 4–6; E = day 7–12.

FIG. 9. As fig. 6. Distribution of types in a group of the heterotransfer P_nD_c (before division). A = day 1; B = day 2–3; C = day 4–6.

cytoplasm no reactivation occurs, and the inserted animal passes through th cell membrane quite soon after transfer.

Our results with enucleated amoebae are in agreement with those obtaine by many other investigators who have studied either the non-nucleate portions of amoebae cut into halves, or truly enucleated amoebae, e.g. Hofer 1890; Willis, 1916; Lynch, 1919; Clark, 1942, 1943, 1944 *a* and *b*. The genera significance of results obtained by enucleation has been reviewed elsewhere (Mazia, 1952; Danielli, 1952 *a* and *b*). It seems likely that many investigators

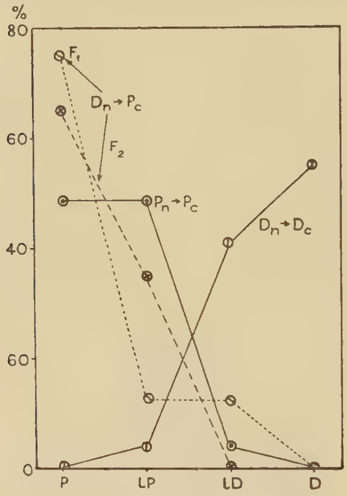

FIG. 10

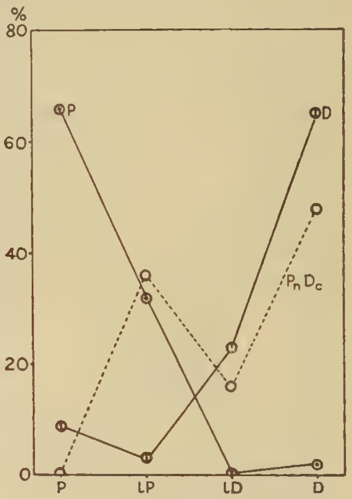

FIG. 11

FIG. 10. As fig. 6. Distribution of types in a group of the heterotransfer D_nP_c, after division. F_1 after the first division; F_2 after the second division.

FIG. 11. As fig. 6. Distribution of types after double nuclear transfer for a group of transfers $P_n \rightarrow D_c$ (before division).

have made unrecorded attempts at nuclear transfer (Robert Chambers, personal communication), but it was not until Commandon and de Fonbrune showed that the transfer must be direct, from one cytoplasm to another, that success was achieved. Exposure to all foreign media so far essayed causes irreversible damage to a nucleus, so that when implanted in a new cytoplasm it is treated as a foreign body. We have attempted to find less toxic media by making injections into the cytoplasm close to a nucleus, and have found none which do not cause irreversible damage when massive injections are made. Large injections of many of these media can safely be made into the cytoplasm at points distant from the nucleus. Our results with nuclear transfers are in agreement with those made by Commandon and de Fonbrune on *A. sphaeronucleus*, so far as reactivation and division of homotransfers are concerned. But detailed comparison is not possible since very few results are available with *A. sphaeronucleus*. No other investigator has studied heterotransfers.

The results obtained here give seven methods of studying the relationship between nucleus and cytoplasm which have not hitherto been exploited. These involve study of (1) reactivation, (2) division after transfer, (3) clone formation after transfer, (4) average nuclear diameter, (5) the position of peaks in nuclear diameter distribution curves, (6) growth of nuclei, and (7) the form assumed when moving. The reactivation which occurs when a cytoplasm is renucleated shows that something is contributed by a nucleus which renders the formation of pseudopods more regular, more frequent and seemingly more purposive. The capture, killing, and digestion of food is also more effective in renucleated animals than in enucleated cytoplasms. This contribution is not species-specific. Brachet (1952) has shown that when amoebae are cut in half the first observable chemical change is a decline in basiphilia, which is perhaps due to loss of pentose nucleic acid. Mazia (1952) had shown earlier that such non-nucleate halves, although still able to incorporate inorganic phosphorus into organic compounds, do so much more slowly than do nucleated halves.

Although division can occur after both heterotransfers $P_n \rightarrow D_c$ and $D_n \rightarrow P_c$, it is much more common with D_nP_c than with P_nD_c. In both cases it is less frequent than with homotransfers. Furthermore, it seems probable that only nuclei from 'fast dividers' can enter into the complete cycle of mitosis and cell division in a foreign cytoplasm. The significance of these facts is not clear. A more decisive result is that, of all the heterotransfers which have been cultured with the object of obtaining a mass culture, only one has in fact given a mass culture. It seems reasonable to conclude that the failure to obtain more mass cultures of heterotransfers is due to a high degree of incompatibility between nuclei and cytoplasms of different species. Thus there is a non-specific essential contribution made by nuclei which is demonstrated by reactivation, and a species-specific contribution which is only made evident by studies of relatively long-term effects.

The study of average nuclear diameters, of the growth of nuclei in foreign cytoplasms, and of the characteristic maxima in curves of distribution of nuclear diameter all testify to a very strong influence of the cytoplasm on nuclear size. This is evident in the first few days after heterotransfer, and the influence of the cytoplasm is the dominant factor once division has taken place. The simplest interpretation of this might be that in these amoebae nuclear diameter is a character, the determinants for which are carried in the cytoplasm. But the observations reported here have been carried on into the F_2 generation only, and it could be contended that the observed determination of nuclear diameter by the cytoplasm represents a purely physiological relationship which would be adjusted over a number of generations to display nuclear dominance. For example, since *A. discoides* is a smaller animal than *A. proteus*, the tendency towards small nuclei in *discoides* and large nuclei in *proteus* cytoplasm, independent of nuclear species up to the F_2 generation, might be taken to indicate merely a physiological adjustment of nuclear size to the volume of cytoplasm encountered on transfer. This point will be taken up in a later communication.

It does not seem to us that the same type of criticism can be levelled against our observations of the form assumed when moving. Indeed, our records show that large and small members of the same species show the characteristic form of the species. Immediately after heterotransfer there is some displacement towards the nuclear type, but after a few days the nuclear influence appears to be lost entirely.

The general conclusion from our results is that there is a high degree of cytoplasmic control over both nuclear size and the form assumed by an amoeba when moving, as demonstrated by studies of animals up to the F generation. Whether this cytoplasmic control can be modified, either slowly or discontinuously, by nuclear activity remains to be investigated. This conclusion contrasts with the results we have obtained, in collaboration with Prof. S. Hörstadius, by the study of nucleated and enucleated cells of developing echinoderm larvae. With the echinoderm material fresh steps in differentiation were found to occur only in nucleated cells. Although enucleated cytoplasms could modify the differentiation of nucleated cells, no change could be detected in enucleated cells themselves. In the developing egg we are dealing with a system which has the normal property of undergoing a series of rapid changes in differentiation, whereas with the amoebae the system is one in which there is a strict maintenance of a given degree of differentiation. Our results are not incompatible with the view that changes in differentiation are nucleus-dependent, whereas maintenance of a given degree of differentiation is primarily cytoplasm-dependent.

The physiological mechanism of mediation and maintenance of cytoplasm-determined characters remains to be discovered. The observations we have made in some theoretical aspects recall the elegant studies made by Lwoff and Chatton of the reproduction and function of kinetosomes, but it seems unlikely that the determinants in amoeba cytoplasm will prove to be readily visible bodies, as is the case with kinetosomes. With these amoebae there appears to be no mating process, no conjugation of nuclei, and no meiotic process. Under such circumstances a large part of the importance of retaining genes on the chromosomes is lost, and it may well be that genes which, in mating cells, would necessarily be chromosome genes, in these amoebae are plasmagenes. The same is true of those differentiated cells which can no longer give rise to mating cells.

In conclusion, it should be mentioned that there are many points of similarity between our results and the observations of Hämmerling (1953) on *Acetabularia*.

We are most grateful to Sister Monica Taylor and Sister Carmela Hayes for their kindness in supplying our initial stock of *A. proteus* and *A. discoides*. The preliminary stages of the work were financed by a grant from the British Empire Cancer Campaign, and the later stages by a grant from the Nuffield Foundation. We are indebted to the Rockefeller Foundation for a gift of microscopes and to the Royal Society for the loan of micromanipulators.

TABLE 1. *The numbers of amoebae reactivated, dividing, and giving clones after nuclear transfer. The figures are for numbers of animals unless in italics, which indicate percentages*

Nature of transfer	*Number in group*	*Number re-activated*		*Number dividing at least once*		*Number giving clones*	
Homotransfers							
$P_n \rightarrow P_c$	69	67	*97*	52	*75*	47	*68*
$D_n \rightarrow D_c$	80	71	*89*	62	*78*	61	*76*
Heterotransfers							
$D_n \rightarrow P_c$	73	64	*88*	25	*34*	0	*0*
$P_n \rightarrow D_c$	86	67	*78*	2	*2*	0	*0*

TABLE 2. *Cases in which reactivation was followed by death without division*

Type of transfer	*Number in group*	*Duration of active life: % living after*		*Proportion cytolysed: % after*		
		3 days	*6 days*	*7 days*	*10 days*	*15 days*
$P_n \rightarrow P_c$	11	70	50	10	20	70
$D_n \rightarrow D_c$	9	80	40	45	60	90
$D_n \rightarrow P_c$	35	70	35	15	30	90
$P_n \rightarrow D_c$	53	26	0	36	80	100

TABLE 3. *The effect of nuclear transfer on the lag* t_l *and the interval between divisions* t_d. *Values of* t_d *and* t *in days. Percentages are of those individuals actually dividing*

Nature of transfer	*Number of individuals observed*	*Fast dividers*			*Slow dividers*		
		%	t_d	t_l	%	t_d	t_l
Unoperated							
P_nP_c	25	*c.* 100	3	0·15	0	. .	. .
D_nD_c	25	75	3·4	0	25	16	3
Homotransfers							
$P_n \rightarrow P_c$	46	75	5	1·7	25	14·4	4·8
$D_n \rightarrow D_c$	57	75	3·2	1·2	25	14	4·4
Heterotransfers							
$D_n \rightarrow P_c$	64	95	4·8	0·5	. .	. .	. .
$P_n \rightarrow D_c$	58	. .	. .	. .	. .	. .	. .

TABLE 4. *A comparison of the effect of transfer of nuclei* (a) *immediately afte enucleation and* (b) *with an interval between enucleation and renucleation*

Nature of transfer	*Immediate transfers*			*Transfers made 24–48 hours after enucleation*		
	Number of transfers	*% reactivated*	*% dividing*	*Number of transfers*	*% reactivated*	*% dividing*
Homotransfers						
$P_n \rightarrow P_c$ . .	69	97	75	8	50	25
$D_n \rightarrow D_c$ . .	80	89	78	6	50	50
Heterotransfers						
$D_n \rightarrow P_c$ . .	73	64	34	9	67	11
$P_n \rightarrow D_c$ . .	79	84	2	15	73	6

TABLE 5. *Average nuclear diameters in heterotransfers at various times after transfer but before division. The averages in cultures of* A. discoides *and* A proteus *are 38·2 μ and 45 μ respectively. Many of the animals divided after transfer. Almost all the animals which divided did so in less than 6 days, so that the figure for 7–9 days represents mainly animals which ultimately died without dividing. The standard deviations diminish after transfer. Thus for* $D_n \rightarrow P_c$ *the initial mean was 35 s.d. 3·9, and after four days the value was 45 s.d. 3·0. For* $P_n \rightarrow D_c$ *the initial mean of 43 s.d. 5·2 changed to 44 s.d. 4·2*

Nature of transfer	*No. of individuals measured*	*Days after transfer*				
		0	1	2	3–4	7–9
$D_n \rightarrow P_c$	52	35	38	42	45	50·5
$P_n \rightarrow D_c$	63	43	44	45	44	. .

TABLE 6. *Values of the maximum diameter* 2r *of nuclei in* D_nP_c *at various intervals after nuclear transfer, averaged for the ten specimens recorded in fig. 7*

Days	0	1	2	3	4	5	6	7
$2r$ in μ . . .	33	37	41	44	45·5	46·5	48	49·5
$r^3 \times 10^{-3}$ . . .	4·5	6·3	8·6	10·6	11·8	12·8	13·8	15·1
Increments in $r^3 \times 10^{-3}$		1·8	2·3	2·0	1·2	1·0	1·0	1·3

TABLE 7. *Average changes in nuclear diameter in heterotransfers* (a) *after transfer of a foreign nucleus and* (b) *after replacement of the first foreign nucleus by a second foreign nucleus. The average nuclear diameters obtained by measuring* 95 *individuals in each parent culture were* 36 μ *for* A. discoides *and* 43 μ *for* A. proteus. *Diameters in* μ

	Diameters of first nuclei				*Diameters of second nuclei*			
Nature of transfer	*Number meas.*	*Just after transfer*	*24 hours after*	*Growth in* μ	*Number meas.*	*Just after transfer*	*24 hours after*	*Growth in* μ
$P_n \rightarrow D_c$.	19	42	44	2	6	38	43	5
$D_n \rightarrow P_c$.	16	36	47	11	1	31	47	16

TABLE 8. *Position of maxima in nuclear diameter distribution curves.* + *indicates frequent occurrence of a maximum;* (+) *indicates occasional occurrence of a maximum;* * *indicates greatest maximum found in a group of animals*

	Nuclear diameter in μ										
Type	32	34	36	38	40	42	44	46	48	50	58
P_nP_c . . .				(+)	+		+*			+	(+)
D_nD_c . . .	(+)	+		(+)	+*						
$D_n \rightarrow P_c$ . . .											
Day . . .											
0	+*			+							
1		+			+		+*				
2				+	+		+*				
3–4 . . .					+		+*		+		
$P_n \rightarrow D_c$ . . .											
0		+		+	+		+*			+	
1				+	+		+*			+	
2		+					+*			+	
3–4 . . .		+	+				+*			+	(+)

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The Micro-anatomy of the Mammalian Epidermis

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SUMMARY

The greater part of the superficial epidermis of mammals is of binary embryological origin, and consists of cells belonging to two distinct division lineages: that of the Malpighian or keratinizing system, and that of the melanocyte or pigmentary system. The melanocyte system can be artificially destroyed, or denied access to the epidermis in development, without impairment of any but the pigmentary activity of skin.

The epidermis as a whole is a reproductively self-contained system, i.e. it is perpetuated by the division of cells that reside within the epidermis itself. It is argued that melanocytes as well as Malpighian cells are squamous in character, and that the functional melanocytes of the basal epidermal layer undergo a characteristic sequence of involutionary changes in the course of moving towards the skin surface to be flaked away. It follows that the entire epidermis, and not merely the Malpighian system, undergoes a continuous process of cellular renewal. Cell-divisions certainly occur in the basal epidermal layer; a number of difficulties of interpretation must be overcome before it can be held certain that divisions occur in more superficial layers as well.

The Malpighian system has a cellular organization: there is no satisfactory evidence for the existence of intercellular cytoplasmic bridges that would endow it with a syncytial character. 'Tonofibrils' probably owe their origin to an artificial coarsening of a fine fibre-protein system within the cytoplasm of prickle cells. Elastic fibres probably play some part in anchoring the epidermis to its substratum; but there is evidence that the epidermis can be freed from the corium by disengaging the downwardly directed processes of Malpighian cells of the basal layer from the concentrated connective tissue ground-substance that forms the inner boundary of the dermo-epidermal interface.

All the natural pigments of mammalian skins are melanins; melanocytes are the only seat of melanin formation, and pigmentary activity is the only function they are known to possess. Branches that arise from the perikarya of melanocytes are so arranged that each ends in close apposition to the superficial pole of a Malpighian cell

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and in some unknown manner causes pigment granules to enter into it. Tyrosine is probably the parental substrate in melanogenesis, and it is unlikely that more than one oxidase is responsible for the enzyme-mediated activities that lead to the formation of melanin.

The fine structure and physiological activity of the Malpighian system varies from place to place on the body: many of the differences which appear to be due (and which it is shown, could have been due) to differences of environment and manner of use are in fact of developmental origin and of 'cellular genetic' status. Such differences are conserved through repeated cell-divisions after the transplantation of particular areas of skin to anatomically unnatural environments. The regional anatomy of the melanocyte system is less complex. The melanocytes of the hair bulbs and of the superficial epidermis, responsible for the pigmentation of skin and hair respectively, are artificially interchangeable. It is therefore probable that they represent purely topographical variants of a homogeneous cellular genetic system. Differences of pigmentation between the variously coloured areas of a patched animal are due to differences between the pigmentary activities of individual melanocytes, and these are perpetuated in cellular heredity; they are not due to differences of structure, density, or distribution.

1. INTRODUCTION

THE epidermis forms an uninterrupted cellular investment to the whole of the outer surface of the mammalian body. In textbooks of histology the epidermis is classified as a 'stratified squamous epithelium': an 'epithelium', because it consists of (or derives from) a uniformly polarized system of contiguous cells so ordered as to bound a surface (see note 1 at end of paper); 'stratified', because certain of the division products of its generative cells remain attached to and superimposed upon the parental layer; and 'squamous', because the ultimate fate of those cells which do not persist as the reproductive lineage is to be flaked away at the free surface. The epidermis is, of course, an avascular structure—a description of uncertain import, for no vertebrate epithelium is vascular—and contains no mesenchymal cells of other than adventitious origin and ephemeral life.

The epidermal epithelium does not present a plane surface to its substratum, the dermis or corium, but is thrown into a pattern of hills and valleys that are exactly complementary in structure to the dermal papillae. The thicker the epithelium, the bolder are these inequalities of depth. In the general body skin of a hair-bearing mammal, the hills are low and rounded, and the valleys correspondingly broad and shallow; the opposite extreme is represented by the horn of the rhinoceros, 'where the greatly elongated and thread-like dermal papillae give it a fibrous texture, leading to the common but erroneous statement that a rhinoceros horn is constructed of thickly matted hairs' (Le Gros Clark, 1952). Whether shallow and rounded or tall and steep, the hills and valleys have a regular and repetitive pattern, as the accompanying text-figures (figs. 1–4) clearly show.

Hairs and a variety of exocrine secretory organs, together comprising the epidermal appenda, arise in development as blind invaginations of the epithelium, and never normally lose their connexion with it. Notwithstanding its apparent perforations by the openings of hair follicles and of the ducts of

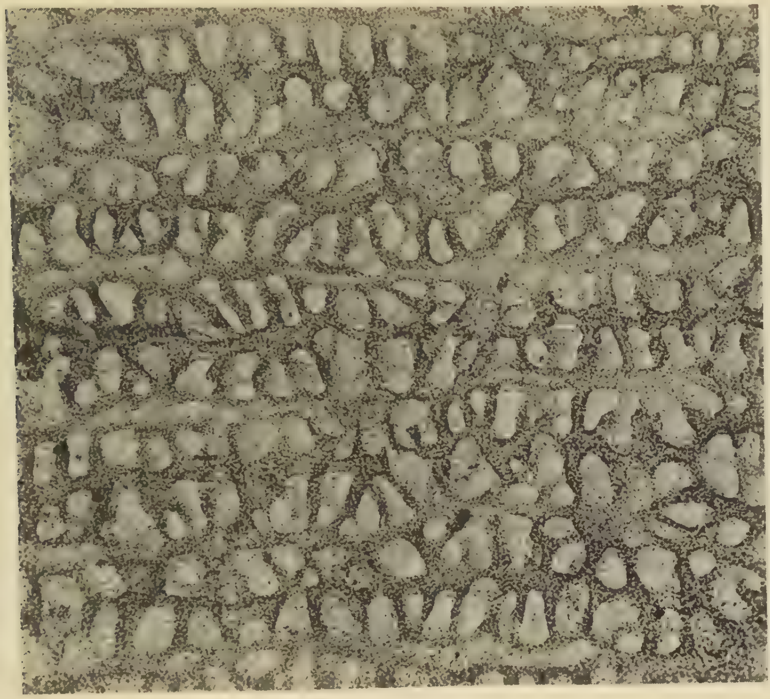


FIG. 1. The pattern of hills and valleys in the epidermis on the pad of the little finger of a human being: the dermal papillae fit into the valleys. Carmalum. ×58.

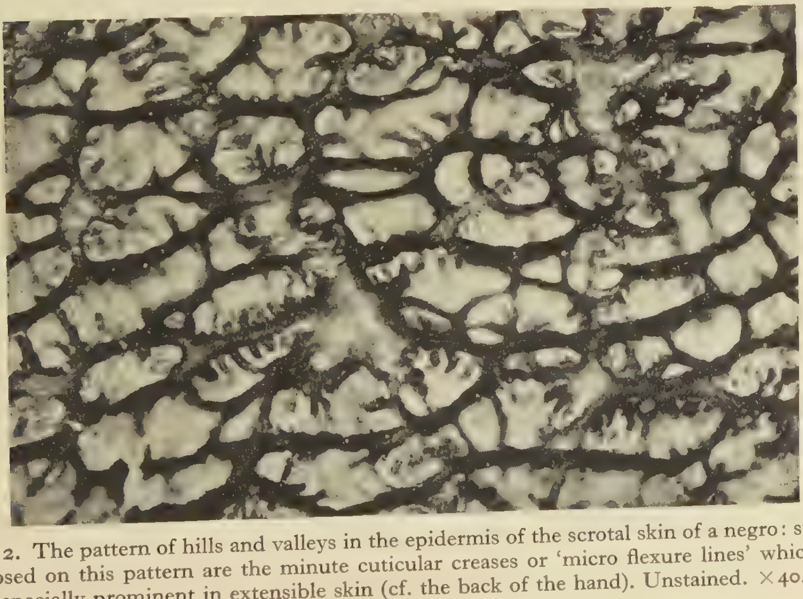


FIG. 2. The pattern of hills and valleys in the epidermis of the scrotal skin of a negro: superimposed on this pattern are the minute cuticular creases or 'micro flexure lines' which are specially prominent in extensible skin (cf. the back of the hand). Unstained. ×40.

glands, the epithelial investment of the body is therefore continuous and complete. It is not known whether the cells responsible for the various exocrine secretions and for forming the hard keratins of hair constitute embryologically distinct lineages, or whether certain cells normally resident in the adult epidermis are capable, given the appropriate stimuli and physical surroundings, of differentiating *de novo* into glands and hairs. The epidermal appenda are to be excluded from the subject-matter of this article, but this conspicuous gap in our knowledge must be borne in mind in reading the paragraphs that deal with the cellular lineages of the epidermis.

In cellular composition, the greater part of the superficial epidermis is of binary origin, i.e. it consists of two entirely distinct and experimentally dissociable division lineages of cells, the members of which have different structures, properties, and embryonic origins. About 85–95 per cent. of the cells (there are no exact figures) derive from the embryonic ectoderm and constitute the *Malpighian* or keratinizing system. The remainder arise from the neural crest (Rawles, 1947, 1948, 1953) and invade the epidermis after 'determination' is complete, i.e. after the epidermis is irrevocably committed to a unique pathway of future differentiation. These constitute the melanocyte, epidermal glial, or *pigmentary* system. In the course of keratinization, the cells of the Malpighian system undergo that regular sequence of cytological changes which gives the epidermis its distinctive pattern of stratification (basal layer, prickle-cell layer, granular layer, *stratum lucidum*, and cuticle); these transformations are so well figured in textbooks of histology that they require no further discussion here. In section 7 it will be argued that the cells of the pigmentary system undergo an analogous sequence of changes, i.e. that certain division products of melanocytes take part in the general outward movement of the epidermal strata to be flaked off at the skin surface, undergoing a regular sequence of cytological transformations as they do so. It may provisionally be assumed, therefore, that the entire epidermis undergoes a continuous process of renewal which makes good the loss of cells by desquamation.

The matters to be discussed in the present article are these: the reproductive self-maintenance of the Malpighian (section 2) and pigmentary (section 7) systems; the nature of tonofibrils and intercellular bridges, in so far as they bear upon the problem of the cellular organization of the epidermis (section 3); the attachment of the epidermis to the corium (section 4); the regional anatomy of the Malpighian (section 5) and pigmentary (section 8) systems; and, lastly, the micro-anatomy of melanocytes, with special reference to their relationships with the Malpighian cells in their immediate neighbourhood (section 6).

2. The Reproductive Self-maintenance of the Malpighian System

Asked to express an opinion on how the epidermis maintains itself reproductively—how it provides for cellular replacement to make good the continuous desquamation of scurf—histologists who were not particularly familiar

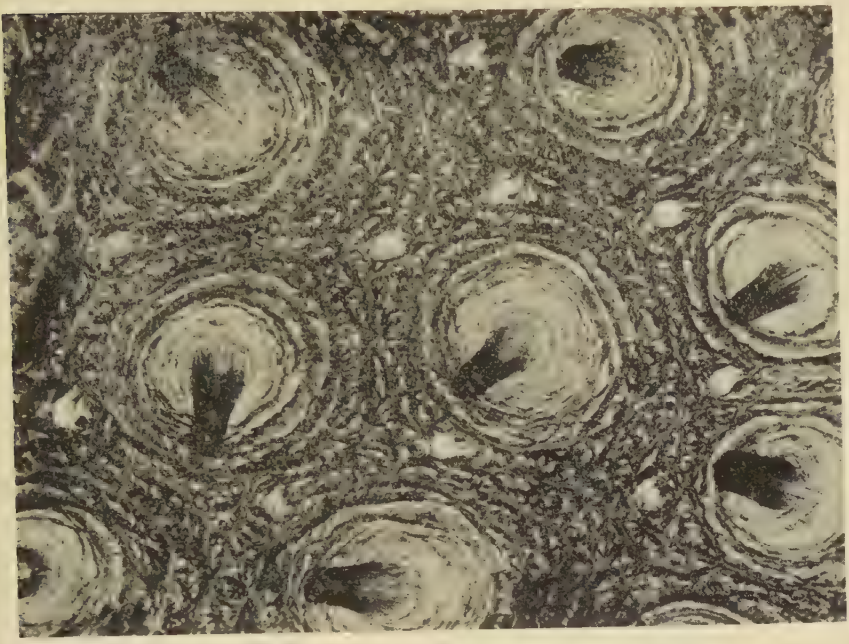


FIG. 3. The pattern of epidermal hills and valleys in the epidermis of the skin of a pig's snout: stout hairs emerge from the centre of the rings of hills, and each system of rings shares an orbit of four or five mucous gland apertures with its neighbours. Unstained. ×20.

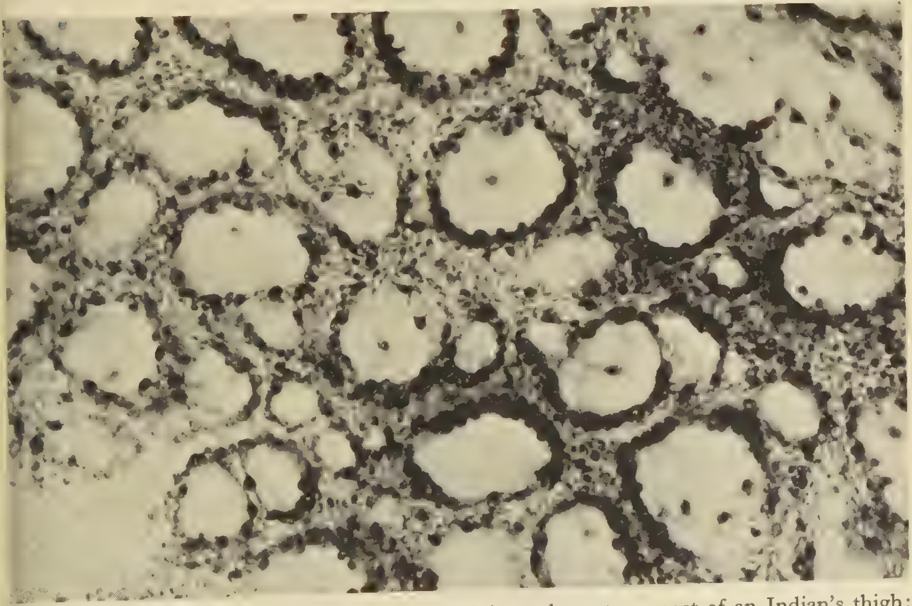


FIG. 4. The pattern of the epidermis removed from the outer aspect of an Indian's thigh: Dopa reaction. See fig. 11 for the detailed structure of the melanocytes. ×118.

with the literature of dermatological research would probably say that the generative cells of the epidermis were wholly contained within the epidermis itself; and would probably agree with Walther Flemming that cell-division took place principally, if not entirely, in the epithelial layer that bounds the dermo-epidermal interface.

The former belief is quite certainly correct, and deserves notice only because of the persistent recurrence of ill-informed regrets that the number of mitoses visible in ordinary histological sections of skin is insufficient to account for cellular replacement. The work of Billingham and Reynolds (1952) and Billingham and Sparrow (1953), although carried out with no such purpose in mind, provides direct experimental proof that the epidermis as a whole—Malpighian cells and melanocytes—is a *reproductively self-sufficient system*, i.e. that the entire epidermis may be regenerated from cells derived from and normally resident within it. In rabbits, 20–25 cm.^{2} of richly keratinized epidermis, 10–15 cells thick, may be regenerated within 15 days from the epithelial cells scraped from the under surface of 2–3 cm.^{2} of dorsal ear-skin epithelium and then 'seeded' over a raw area on the chest stripped down to a plane well below the depth of the deepest hair follicles. There is, therefore, no need to suppose that the transformation of adventitious mesenchymal cells makes up a hypothetical deficit of epidermal cell number, and the 'lymphocytes' to which Andrew and Andrew (1949) have recently allotted this gratuitous function are in fact 'clear cells', i.e. the perikarya of melanocytes (Billingham and Medawar, 1953). Nor have careful modern studies of the mitotic activity of the epidermis given any reason to suppose that the epidermis is so improvident as to exist in a state of habitual mitotic debt.

The second belief, that the epidermis is constantly regenerated by division of cells in the basal epithelial layer, requires some critical attention. That regeneration is 'from within outwards' is denied by no one, and Leblond (1951) has provided a neat autoradiographical mapping of the outwardly directed movement of epidermal cells in new-born rats injected with $_{14}$C-labelled carbonate. The average time taken for a complete cycle of regeneration, i.e. the renewal time of the epidermis, will naturally vary from place to place; the traditional figure of 7 years must now give way to Storey and Leblond's (1951) estimate of 19·1 days for the plantar epidermis of adult rats at a temperature of $27 \pm 3^\circ$ C. But in what layer of the epidermis do divisions principally occur? From Flemming's day onwards, orthodox opinion has had it that the great majority of cell-divisions, if not all, occur in the basal layer, and our newer knowledge of the exacting oxygen requirements of mitosis (Bullough and Johnson, 1951; cf. Medawar, 1947) makes it difficult to credit the belief that cell-division can be initiated at the oxygen tensions which presumably prevail in the middle of a stratified epithelial layer (see note 2). Nevertheless, Thuringer (1924, 1928, 1939) has long insisted that mitoses are more frequent—Cowdry and Thompson (1944) find them to be insignificantly less frequent—in the prickle cell than in the basal layer. Before accepting these results at their face value, two sources of error, cogently analysed by

Hanson (1947), deserve more attention than they appear to have received. The first is that these studies have been made upon a *thick* epidermis, distinguished by a pattern of closely packed, steeply rising hills and valleys. Sections of the epidermis that cut perpendicularly across the skin surface must therefore, in many regions, be parallel to a plane tangential to the dermo-epidermal interface. In such regions the section is *anatomically* horizontal, and cells that appear to be in a superficial epidermal layer may in fact belong to the basal layer (cf. Billingham and Medawar, 1953). Secondly, the use of colchicine in the analysis of mitotic frequencies denies the investigator the chance of identifying the prophase of mitosis, and Hanson points out that cells which have begun to divide in the basal layer may have entered a more superficial layer before division is complete. It is relevant to this interpretation that mitosis, once begun, may be completed at very low oxygen tensions, perhaps anaerobically (Bullough and Johnson, 1951). These are objections of some weight, and until they have been removed, the orthodox opinion that mitoses are initiated in the basal layer must be held to prevail.

The interpretation which Cowdry and Thompson (1944) read into their findings is that some of the descendants of epidermal cells which have arisen by division in the basal layer may not have lost their power to divide when they have reached the prickle-cell layer. In other words, the division lineage begins in the basal layer and peters out superficially. But this is not the only construction that may be put upon the possibility that mitoses occur in the prickle-cell layer, and it must be emphasized that there are no valid grounds whatsoever for supposing that prickle cells, if indeed capable of division, constitute a lineage of cells *sui generis*, i.e. are cells of a histogenetically distinct type that perpetuate themselves in the intermediate strata of the epidermis. Prickle-cells arise by a cytological transformation of basal layer cells, and the simplest experimental devices can cause them to be manufactured at will. The epidermis of the general trunk skin of the rabbit consists of little more than basal layer cells bounded by a delicate cuticle; but if it is caused to proliferate by shaving, or by the application of turpentine or chloroform or some other irritant, it thickens within a few days and acquires a prickle-cell layer, stratum granulosum, and many-layered cuticle. Left to itself, it subsides slowly into its original quiescent and relatively undifferentiated state. The formation of a prickle-cell and granular layer is merely incidental to the modified sequence of keratinizing transformations that distinguish the naturally thick or artificially thickened and mitotically active epithelia from the comparatively indolent epithelium of hair-bearing skin.

3. TONOFIBRILS AND INTERCELLULAR BRIDGES: THE CELLULAR ORGANIZATION OF THE EPIDERMIS

The 'prickle-cell layer' of ordinary histological sections is composed of more or less isodimensional cells which are distinguished by the possession of a radially orientated system of very fine fibrillae. These are Heidenhain's

Tonofibrillen, and it is an orthodox and long-standing belief, firmly upheld by Leblond (1951), that tonofibrils pass without interruption from cell to cell, so forming a fibrous continuum that is superimposed upon the cellular ground plan.

Tonofibrils have most of the stigmata of what are commonly, but not always lucidly, described as 'artifacts'. Their boldness of definition varies with the properties of the fixing agent, being least with non-precipitants. They are invisible within enzymically dissociated cells, whether living or fixed. Chambers and Rényi (1925) failed to distinguish them within living human epidermal cells separated by micro-dissection. Electron microscopy of very thin sections of human skin fixed in osmium tetroxide gives only the most fitful evidence of tonofibrils (Pease, 1951); indeed, the fibrous meshwork within the cytoplasm of prickle cells is isotropic, and too fine-grained to be resolved by ordinary microscopy (Adolph, Baker, and Leiby, 1951). Fibrillar structures are invisible in cultivated human epidermal cells examined by phase contrast microscopy (Lewis, Pomerat, and Ezell, 1949) except in regions of the colony in which the cells may have been subjected to forces tending to pull them apart—an exceptional case, the import of which will become apparent in later discussion. But it would be frivolous to dismiss tonofibrils as artifacts. All histological appearances are artifacts of one sort or another, i.e. represent the consequences of optical, mechanical, or chemical transformations of the living system. An artifact is only mischievous if one fails to inquire into the credentials of the transformation process, or assumes that no such process has taken place. It is likely that intracellular tonofibrils (like neurofibrils and myofibrils) are artifacts in the sense that they represent artificial coarsenings, to the level of microscopical visibility, of an underlying fine structure of orientated fibrous protein molecules. It is therefore to the point that treatment of the more deeply situated epidermal cells with 50 per cent. urea (and other less heroic solvents) yields a bulky fibro-gelatinous extractive from which a protein matter, 'epidermin', may be precipitated by one-fifth saturation with ammonium sulphate: X-ray diffraction analysis shows that epidermin belongs to the same structural family as keratin, myosin, and fibrinogen (Rudall, 1946, 1947, 1953). Such a fibrous protein may already have that orientation within the cells of the stratum spinosum which favours its precipitation into microscopically visible, birefringent fibrils. Alternatively, the various operations associated with removing and fixing skin may set up stresses which impose such an orientation upon an otherwise isotropic fibre system. The fact that dissociated epidermal cells, exempt from such forces, reveal no tonofibrils upon fixation, and that fibrillary appearances in cultivated epidermal cells are probably symptoms of the action of distortional forces, inclines in favour of the second possibility.

Intercellular bridges, or the appearances so described in orthodox histology, are probably artifacts of a cruder character. The idea that they represent extensions of tonofibrils across inter-cellular space is difficult to reconcile with the fact that, examined in groups of three or four, the borders of epidermal ells are in fact contiguous. Neighbouring epidermal cells are so firmly united o each other that they tear and break more easily than they pull apart; but trands appear when attempts are made to separate them (Chambers and Rényi, 1925). These strands do indeed represent connexions between cells, out Chambers and Rényi gave no evidence for supposing that they raverse cellular interfaces. Their appearance of doing so in orthodox sections s at least partly due to the fact that interfaces between neighbouring prickle cells are very difficult to identify; when examined by electron microscopy, it is clear that the interfaces subdivide the so-called inter-cellular spaces, and that the 'intercellular bridges' are in fact in*tra*cellular structures formed by the withdrawal of cytoplasm from the surface of the cell (Pease, 1951).

Melanocytes have a non-fibrillar cytoplasm; and although the cytoplasm tends to collapse around the nucleus after fixation, its properties are not such as to create the appearance of bridge-like structures uniting them to the Malpighian cells around.

4. THE ATTACHMENT OF THE EPIDERMIS TO THE CORIUM

The epidermis of normal skin is very firmly united to the underlying corium, and cannot be separated from it by ordinary mechanical means. On the other hand, epithelium that has grown outwards from small skin grafts across granulation tissue (or inwards from the edges of an extensive wound) has an attachment that amounts to little more than mere apposition; indeed, a thick sheet of epithelium which has regenerated from an epidermal cell seeding (see above) may be lifted from its substratum by so simple a means as injecting air between the two layers with a hypodermic syringe (Billingham and Reynolds, 1952).

Very many attempts have been made by microscopists to seek for fibres or strands at the dermo-epidermal interface which look as if they might serve the purpose of anchoring the epithelium down. Earlier work has been reviewed by Herxheimer (1916) and Szodoray (1931), and we may agree with Szodoray that three distinguishable fibre systems may be concerned: (*a*) elastic fibres, particularly those finer fibres which are concentrated into a sub-epithelial plexus; (*b*) the fine downwardly directed extensions of the cytoplasm of basal-layer cells—presumably variants of the filamentous strands visible at the periphery of cultivated epidermal cells (Lewis, Pomerat, and Ezell, 1949); and (*c*) the reticulum of argyrophil fibres, immediately below the basal epidermal layer, described by Friboes in 1920 and by many others since. Odland (1950) presents convincing evidence that these argyrophil fibres form a continuous meshwork, and that the appearance of free bulbous endings described by earlier workers is the consequence of an optical illusion. Inasmuch as neither elastic fibres (Dick, 1947) nor argyrophil fibres (Odland, 1950) nor, indeed, fibres of any sort (Pease, 1951) actually penetrate between the basal layer cells of the epidermis, the only crudely mechanical interpretation of the attachment must be that the downwardly directed extensions of basal layer cells are either gripped in the interstices of the argyrophil reticulum, as Odland supposes, or are embedded in the connective tissue ground substance in which the reticulum itself lies. The idea of a fibrous attachment is therefore not necessarily at odds with the demonstration by Baumberger, Suntzeff, and Cowdry (1942) that the epidermis may be *reversibly* detached by treatment with N/3 ammonia solution or by heating to a temperature between 49° and 50° C. Felsher (1946), studying the ease of separation of the epidermis as a function of the pH of the solution in which skin is immersed, formed the opinion that separation was most effective at that pH at which collagen is most swollen and presumably least cohesive. Something should therefore be said of the properties of the 'basement membrane', using the term to refer to 'the hyaline or optically homogeneous material which gives the impression of being dissectable, as if it were a sheet of material interposed between a cellular surface and the underlying connective tissue' (Gersh and Catchpole, 1949). Most histologists, following Szodoray (1931) and Pautrier and Woringer (1930), believe that the basement membrane has been dispossessed of its claims to independent existence by the discovery of the argyrophil reticulum in the position which it has been held to occupy; but we may reasonably ask whether the general ground substance of the connective tissue of the dermis has any such special properties in the neighbourhood of the dermo-epidermal interface as would give the appearance of an anatomically defined, sheet-like membrane, after certain histological treatments. Gersh and Catchpole believe that the sub-epidermal basement membrane is qualitatively similar to the general ground substance: its most characteristic ingredient is a glycoprotein which is concentrated, gel-like, and probably polymerized, in the zone immediately below the epithelium. The basement membrane is quickly regenerated after inflammatory tissue destruction and after the migration of skin epithelium across the surface of a wound; in all probability, therefore, it is not a sufficient cause of the adhesion of the epidermis.

There is certain ancillary evidence for supposing that elastic fibres play a necessary part in anchoring the epidermis down, in spite of the good anatomical reasons given by Dick (1947) for supposing them to be of minor importance. Elastic fibres are absent from the substratum of that freshly migrated epithelium which, as we have seen, is so very weakly attached (Mandl and Rabinovici, 1946); they are also said to be congenitally absent from the dermal papillae in the affection *epidermolysis bullosa* (see Sutton and Sutton, 1939), which is distinguished by the extraordinary weakness of the dermo-epidermal union and the tendency for blisters to form between the two layers. Furthermore, solutions of commercial trypsin quickly and cleanly cleave the epidermis from the dermis when applied in such a way as to gain access to the junction between the two (Medawar, 1941); since purified tryptic enzymes have no such effect, cleavage may well be due to the action of the pancreatic elastase described by Baló and Banga (1949, 1950). The problem is worthy of further investigation.

Orthodox histological analysis makes it clear that the connective tissue res of the upper reaches of the corium, and particularly of the dermal pillae, are finer, more openly packed, and (by ordinary criteria) 'younger' an the stout, densely packed, mature fibres of the deeper corium. The fference may be due to the fact that the finer, more superficial collagen res are being continuously broken down and generated anew—an inter- retation which could be tested by autoradiography, for the mature collagen res of the dermis and of tendons are perhaps the most indolent of all the rmed elements of the adult body in their rate of atomic turnover (Neuberger, errone, and Slack, 1951).

5. The Regional Anatomy of the Malpighian System

It is a matter of common observation that the epidermis (and the skin enerally) varies in its properties from place to place on the body. In the human eing, for example, the epidermis of the heel and sole is thick, fully stratified, itotically active, and bounded by a many-layered cuticle. The generative pithelium of the nail is distinguished by the formation of a densely packed, on-scurfy cuticle of 'hard keratin', using that term in its special technical ense (Giroud, Bulliard, and Leblond, 1934). The epithelium of the cornea eratinizes in such a way that the entire epithelial layer is almost perfectly ransparent; the skin of the face has a much denser population of melanocytes han, for example, the skin of the thigh (see below); and so on.

Differences between the properties of the skin of various areas of the body ay arise in one or both of two ways. The first type of difference is that which caused by a difference of environment or of manner of use. If only one arm f a white human being is exposed to sunlight, it will become more darkly pig- ented than the skin of the symmetrically opposite area of the other arm; if ne hand is for any reason more chafed or irritated than the other, it will cquire a thicker epidermis; if skin that would otherwise be smooth is epeatedly creased, ectopic flexure lines will form, as they do across the fore- ead, between the eyebrows, or beside the angles of the mouth. In all such ases we have to do with differences of purely environmental origin (see note). Differences that arise in this way do not persist indefinitely after the with- rawal of the stimulus that called them into being; protected from sunlight, he arms will re-acquire their urban pallor; skin thickened by chafing will thin ut when the source of irritation is removed, and ectopic flexure lines would resumably disappear if the features were to be kept in a state of unvarying omposure.

Inasmuch as skin is of all organs the most accessible to the impress of the nvironment, it would be tempting to suppose that much of its finer detailed ifferentiation was the outcome of an adaptive adjustment to differences of unction or of manner of use. It is therefore of some interest that differences hat arise in this way are the exception rather than the rule. Consider, for xample, the thick, mitotically active epidermis of the sole of the foot: might t not be said to owe its distinctive properties to the chronic chafing and echanical stress which soles of feet are habitually subjected to? Exactly the

same question, *mutatis mutandis*, might be asked of the flexure lines of t
palm of the hand, which might so easily represent the engravery of everyd
usage. To both these questions the answer is the same: they could be form
in this way, but, as it happens, they are not. The existence of corns an
callosities in the one case, and of ectopic flexure lines in the other, is eviden
enough that they *could* be formed by adaptive differentiation in response
use—i.e. is evidence that if human beings were born with smooth skin on t
palms of their hands, and with an epidermis no thicker on the sole than els
where in the body, then ordinary usage would faithfully reproduce t
thickenings and flexure lines that we are familiar with. That they are *not*
fact formed in this way is open to embryological and experimental proof.
the first place, thickened soles and palmar flexure lines arise earlier in develo
ment than would be possible if their formation represented an adapti
response within each lifetime to peculiarities of use. Human beings, li
guinea pigs, are born with a thicker epidermis on their soles than elsewhe
on the body; the epithelium of the foot-pads of mice is recognizably differe
from that of the trunk by the eleventh day of gestation (Hanson, 1947); t
more prominent palmar flexure lines are visible in the 12-weeks-old huma
foetus. In the second place, the mitotic activity and distinctive keratinizatio
of the sole of the foot represent differences that are inherited in somat
cellular reproduction. If sole-of-foot epithelium is transplanted to a region
the skin where it is protected from its normal stresses of use, it still preserv
its *differentia* with such distinctness that the two types of epithelia at t
margins of the graft can be distinguished cell from cell (fig. 5; see Billingha
and Medawar, 1948, *a* and *b*). It is the same with palmar flexure lines, f
palmar flexure lines (unlike those of ectopic formation) do not change or di
appear when, as a result of injury or in course of surgical repair, the skin bea
ing them is so displaced as to be subjected to altered patterns of creasing an
folding (see note 4).

What is perhaps unexpectedly true of thickened soles and 'natural' flexu
lines is all but self-evidently true of the properties that distinguish t
epithelia of the claw (fig. 6) or tongue (figs. 7 and 8: see Billingham an
Medawar, 1948 *a* and *b*, 1950*a*), the vagina (Krohn, *unp.*), and the corne
The differences between these several epithelia are indefinitely conserved aft
heterotopic transplantation, and do not owe their maintenance throughou
repeated cell-divisions to the physiological or anatomical peculiarities of th
environments in which they normally reside. A thin sliver of corneal epitheliu
may multiply its area fiftyfold when transplanted to a richly vascular area pre
pared from the skin of the chest, but its peculiar transparency and mode o
keratinization are not appreciably interfered with; conversely, ordinary trun
skin transplanted into the cornea loses none of its distinctive propertie
(Billingham and Medawar, 1950*b*).

The greater part of the regional differentiation of the skin therefore repre
sents, not functional adaptation, but the closing stages of embryonic develop
ment. The difference between a cell in sole-of-foot and general body ski

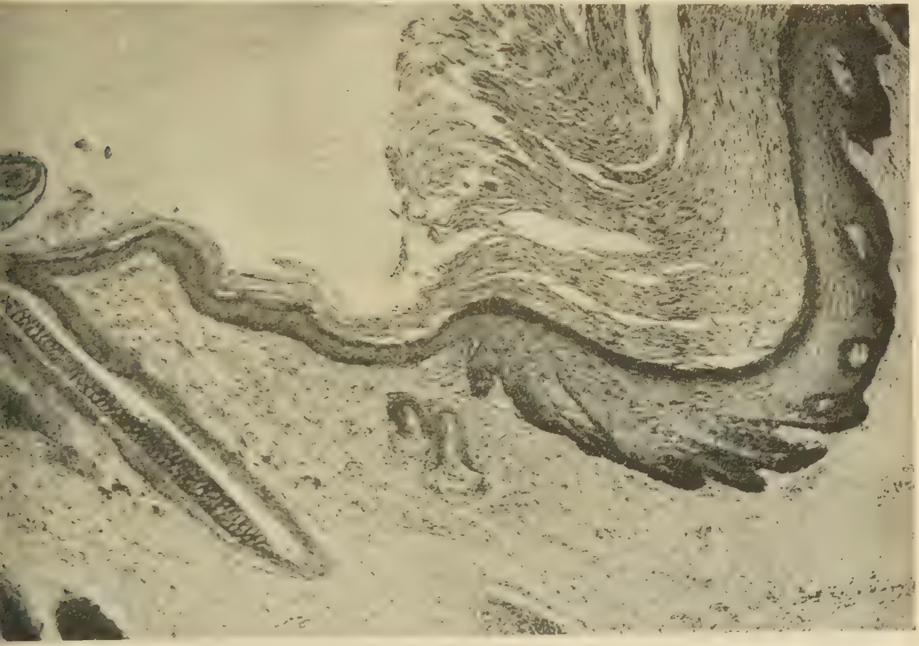


FIG. 5. A transverse section through the edge of a disk of guinea-pig's sole-of-foot skin about 2 months after its transplantation to the skin of the chest. Note the abrupt transition between the relatively quiescent hair-bearing epithelium of the chest and the thickly keratinized epithelium of the sole. There is no intermixture of the cells of the two types, and no sign of any interaction between them. Ehrlich's haematoxylin and eosin. ×88.

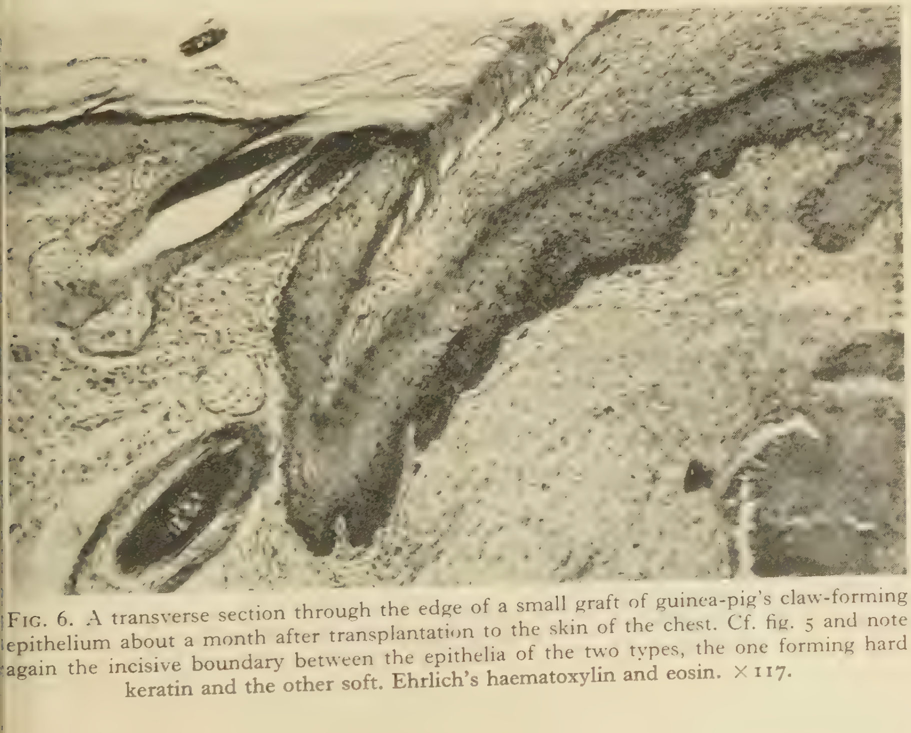
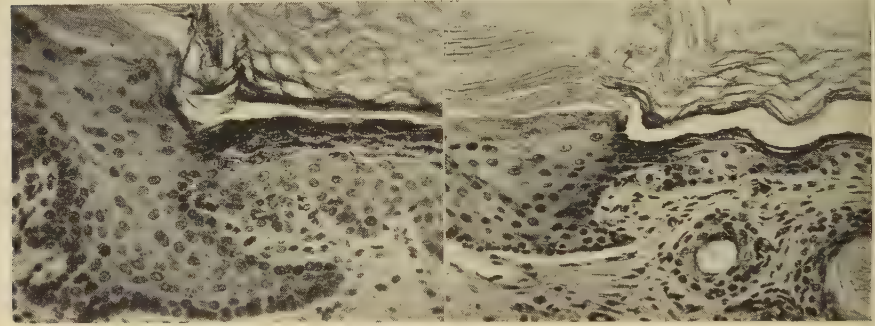


FIG. 6. A transverse section through the edge of a small graft of guinea-pig's claw-forming epithelium about a month after transplantation to the skin of the chest. Cf. fig. 5 and note again the incisive boundary between the epithelia of the two types, the one forming hard keratin and the other soft. Ehrlich's haematoxylin and eosin. ×117.

epithelium is of the same *kind* as that which distinguishes a thyroid cell fro a neurone, or an osteoblast from a melanocyte: it is of developmental origi and it is perpetuated in somatic cellular heredity. Although of the same kin it is, of course, a difference of altogether lesser degree, because the sever epidermal epithelia have a comparatively recent common ancestry in th genealogy of cells that arise by division of the zygote. 'The epidermis' (li 'the fibroblast') is therefore not a cellular species but a cellular genus, and is worth while remembering that the anatomical description of developme is not complete until the several species of each such genus, representing th



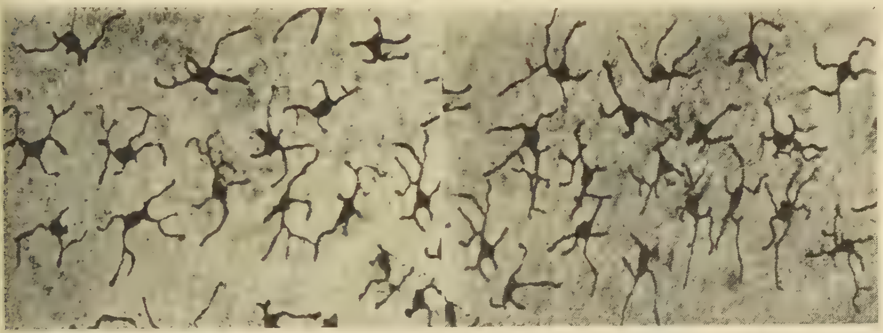
FIGS. 7 and 8. Transverse sections through the edges of small grafts of guinea-pig's tongu epithelium about 2 months after transplantation to the skin of the chest. Notice in fig. 7 th abrupt disappearance of the stratum granulosum as ordinary trunk-skin epidermis gives wa to the thicker epithelium of the tongue; fig. 8 has been chosen to illustrate the equally sudde change between the cuticles produced by the two types of epithelia. Ehrlich's haematoxyli and eosin. × 150.

topmost branches of the tree of differentiation, have been distinguished an duly classified. It may be argued that, in their attempts to propound a causa theory of development, embryologists might find it more rewarding to stud these closing stages of differentiation than the grander but less accessibl dichotomies of the cellular lineage that occur during neurulation and th earliest stages of organogenesis.

6. THE MELANOCYTE: MELANOGENESIS

The melanocyte (see note 5) is the only seat of pigmentary activity in th epidermis, and pigmentary activity is the only special function that th melanocyte is known to possess. No matter what its other functions, if any prove to be, none is indispensable; for some epidermal epithelia lack melano-cytes naturally (see below); others may be denied them by preventing thei immigration from the neural crest (Rawles, 1947), or deprived of them b treatments such as freezing which are relatively innocuous to cells of th Malpighian system (Taylor, 1949; Billingham and Medawar, 1952). Epitheli lacking melanocytes are without exception unpigmented, but do not appea to be at a disadvantage in any other respect (Rawles, 1953).

The melanocyte system is not epithelial in character, but disperse. Its constituent cells lie regularly scattered in the basal layer of the epidermis, with Malpighian cells between them (figs. 4, 9, and 10; see note 6). Each melanocyte consists of a perikaryon which, being slightly larger than a Malpighian cell of the basal layer, abuts just perceptibly below the general level of the dermo-epidermal interface; the branches which arise from it generally run for a variable distance between dermis and corium before, multiplying by secondary branches, they turn outwards to travel between the cells of the basal layer (fig. 11). Each branch ends in a flattened swelling upon the superficial pole of a Malpighian cell, and each melanocyte of the basal layer has a domain or *Lebensraum* with itself at the centre and, around it, the Malpighian cells upon which its branches end. Melanocytes are so distributed that neighbouring domains are contiguous; the branches of neighbouring melanocytes sometimes fuse (figs. 9 and 10), but fusion is not the general rule.



FIGS. 9 and 10. Melanocytes shown up by the Dopa reaction in a 'hill' region of the epidermis from a pale red area of guinea-pig's trunk skin: the epidermis has been removed enzymically from the corium, treated with the Dopa reagent, and mounted as a whole for inspection from the inner (dermal) side. Melanocytes are more spread out in a thin epidermis, such as this, than in a thicker. Anastomoses between branches of neighbouring melanocytes can be seen in both photographs. Malpighian cells, lightly inoculated with rather transparent red melanin granules, are vaguely discernible in the background: they are completely Dopa negative. × 200.

The numbers of melanocytes vary from area to area, and, within corresponding areas, from one individual to another. The unpublished estimates so far collected by G. Szabo give a mean of 750 melanocytes per mm.^{2} of dermo-epidermal interface in plane projection for skin from the outer aspect of the thigh of white human beings (eight estimates ranging from 435 ± 27 to $1{,}015\pm55$); for the skin of the cheek and forehead, four estimates ranged from $1{,}956\pm199$ to $3{,}650\pm100$ (mean 2,500). There are, therefore, about three times as many melanocytes in the skin of the face as in the skin of the thigh. Although 10,000 is probably a fair estimate of the number of Malpighian cells per mm.^{2} of the dermo-epidermal interface, the convolutions of the epidermis will ensure that a decidedly larger number is present in that area which appears as 1 mm.^{2} in plane projection. The ratio of the numbers of

Malpighian cells in the basal layer to the number of melanocytes can nevertheless be taken, as a purely provisional figure, to be about 20:1 in thigh skin and 6:1 in the skin of the face. Other computations of the numbers of melanocytes appear in section 8.

Most of the foregoing description of the micro-anatomy of the melanocyt system is based upon the interpretation of Billingham (1948) and Billingham and Medawar (1948*a*, 1953), who have relied mainly upon the study o anatomically undisturbed and living preparations of sheets of 'pure epidermis separated from the underlying connective tissue by the action of pancreatic enzymes (see above). It is exceedingly difficult to make head or tail of the micro-anatomy of the melanocyte system when using transverse sections of skin, and for this reason, no doubt, it is almost invariably misrepresented in textbooks of histology. Paradoxically enough, it is the melanocyte of feebly pigmented skin that stands out most clearly in orthodox transverse sections: as Masson has long insisted (1948 *a* and *b*), the perikaryon of such a melanocyte appears in transverse section as a 'clear cell', and in spite of cavillings that are still to be found in recent literature, this interpretation may now be held to be established beyond reasonable doubt (Billingham and Medawar, 1953).

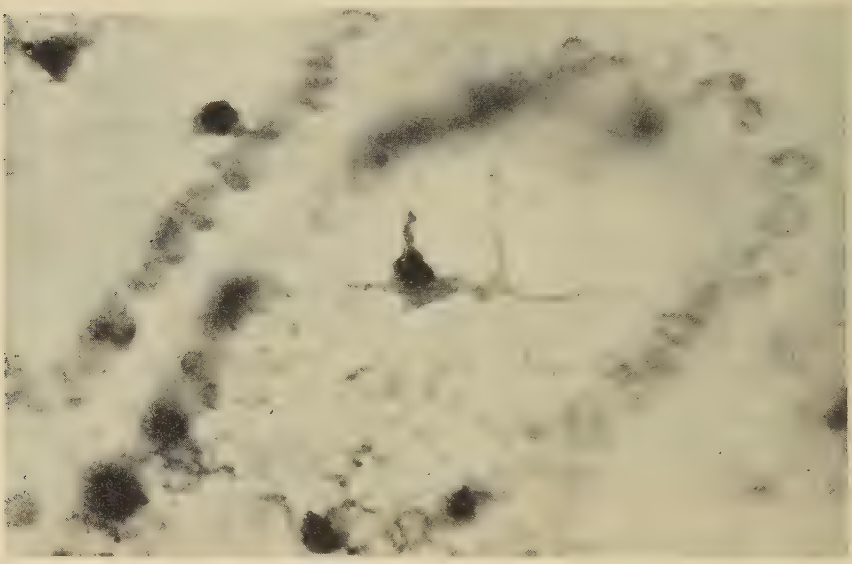


FIG. 11. A melanocyte from a valley area of the preparation illustrated by fig. 4: Dopa reaction. Notice how, in a flat area of epithelium, the branches reach out towards Malpighian cells across the dermo-epidermal interface. The Malpighian cells, just discernible in the background, are lightly inoculated with melanin granules, but are Dopa negative. × 618.

The cytoplasm of melanocytes in a state of pigmentary activity is congested with melanin granules. These, however, do not always serve to show up their outlines distinctly, for the Malpighian cells in their neighbourhood also contain melanin granules. The manner in which melanin granules formed vithin melanocytes are caused to enter Malpighian cells is still uncertain; it nay be a passive inoculation or an active ingestion, for pinocytosis has been lescribed in Malpighian cells in tissue culture (Lewis, Pomerat, and Ezell, 949). However that may be, the transfer of a formed secretory element from ne cell directly into another is a process unusual enough to deserve a special ame, *cytocrine* activity (Masson, 1948*a*). When the transfer is first effected, he melanin in the Malpighian cell is concentrated into a 'cap' at its superficial ole, corresponding to the position at which the branches of the melanocytes nd (Billingham, 1948); but as the Malpighian cell moves outwards in the ourse of desquamation, the melanin granules become more evenly distributed nd, apparently, somewhat sparser.

Something should now be said of the current views on the biochemistry of pigment formation. Although injected flavins may be incorporated into the hairs of albino rats (Haddow, Elson, Roe, Rudall, and Timmis, 1945), and white human skin is said to acquire a yellow tinge after over-indulgence in eating carrots, the natural pigments of mammalian hair and skin are *melanins*—variously coloured matters, usually conjugated with proteins, formed by polymerization of the oxidation products of phenols. Interpretation of their mode of origin was long dominated by the opinion of Bloch (cf. Bloch and Schaaf, 1925; Bloch, 1927, 1929) that melanins are formed by the oxidation of 3:4 dihydroxyphenylalanine (hereafter 'Dopa') by a specific intracellular enzyme, Dopa-oxidase. There was good indirect evidence in support of Bloch's opinion. Tyrosinases, though widespread in lower animals and plants, had not been identified in mammalian tissues, for Durham's (1904) belief that they were present in the skin of foetal rabbits had not been confirmed by Onslow (1917). The incubation of frozen sections of skin in suitably buffered solutions of Dopa—but not of tyrosine—had been shown to lead to the formation of Dopa melanins within the pigmentary cells of the epidermis. (This, Bloch's 'Dopa Reaction', has been the foundation of much exact work on the fine anatomy of the pigmentary system, for within the epidermis the Dopa reaction is strictly confined to melanocytes: cf. figs. 4 and 9–11). Moreover, Dopa had been isolated by Raper (1927) from the products of the oxidation of tyrosine under the influence of mealworm tyrosinases. But the opinion is now firmly taking shape that tyrosine is the natural starting-point of melanin formation in mammalian skins, and that a tyrosinase alone is competent to account for the entire sequence of enzyme-mediated oxidative changes that anticipate the polymerization of melanin precursors. Raper's important series of papers in the *Biochemical Journal* constitute the foundation of the chemical theory of melanogenesis: Dopa is an early intermediary in the oxidation of tyrosine, and 5:6 dihydroxyindole probably a later (see Beer, Clarke, Khorana, and Robertson, 1948; Mason, 1948, 1953; Bu'Lock and Harley-Mason, 1951). Tyrosinases have been extracted from a variety of mammalian melanotic tumours (Hogeboom and Adams, 1942; Greenstein and Algire, 1944), and from the skins of rats (Spoor and Ralli, 1944) and mice (Foster, 1951); they have been revealed histochemically in the skins of white human beings after activation of the melanocytes with thorium-X (Becker, Fitzpatrick, and Montgomery, 1952) and both histochemically and manometrically in the skin of foetal guinea pigs (Foster, 1952). Lerner and Fitzpatrick (1950, 1953), to whom we are indebted for admirable reviews of the theory of melanin formation, argue that, inasmuch as a Dopa-oxidase will catalyse the oxidation of tyrosine, and a tyrosinase that of Dopa, one enzyme or the other is probably of supererogatory status. 'Tyrosinase' enjoys the rights of prior definition and tyrosine is the prior substrate. That there is one phenoloxidase rather than two or more is also the opinion of those who have studied the properties of tyrosinases of other origins (Nelson and Dawson, 1944).

7. THE REPRODUCTIVE SELF-MAINTENANCE OF THE PIGMENTARY SYSTEM

Epidermal melanocytes, it has already been argued, are a reproductively closed or self-sufficient system, i.e. they are perpetuated by the division of cells that reside in the epidermis itself, and there is no evidence that their numbers are made good by immigrants from the corium. Division stages, though far from easy to see, have been adequately figured by Billingham (1948; see also Masson, 1948*a*; Pinkus, 1949; Becker, Fitzpatrick, and Montgomery, 1952). Dividing cells do not lose their pigmentation during fission, although cytocrine activity may well be in abeyance; there is therefore no reason to suppose that the generative cell of the pigmentary system is a not-yet-pigmented 'melanoblast' which differentiates into a pigmentary melanocyte that is no longer capable of dividing.

What is the fate of melanocytes in the epidermis? There are two possibilities. Either (*a*) each daughter cell retains its pigmentary activity, its power to divide, and its position in the basal layer, so that divisions occur only to make good the deficit of accidental loss; or (*b*) the melanocyte, as Redslob (1922) and Masson (1948*a*) have suggested, is a squamous cell—in the sense that some of its division products participate in the general outward movement of epidermal cells, eventually to be flaked off at the skin surface. If the latter interpretation is correct, it follows that the division lineages of melanocytes and Malpighian cells are formally identical: only a certain proportion of division products remain in the basal layer to constitute the germ line; the rest, 'expendable' cells, make up the perpetually transient population of the more superficial layers of the epidermis.

Billingham and Medawar (1953) have made a systematic investigation of these alternative hypotheses; their evidence is too lengthy to be summarized here, but their conclusions, which support Masson's conjecture, are as follows. Melanocytes are squamous cells, and the transformations which they undergo in course of involution and desquamation are as orderly and well-defined as those of Malpighian cells. The first stage of involution is the discharge of all pigment granules, accompanied by the complete loss of Dopa reactivity and some reduction of cell size. The effete melanocytes now occupy a position between the basal layer and the stratum granulosum, still in an evenly spaced and two-dimensional ordering, and corresponding one-to-one in number with the melanocytes that remain behind in the basal layer. Individual cells have a rather desiccated, refringent appearance, but each still retains a Feulgen-positive nucleus and a complement of wiry but fully extended dichotomizing branches. In this condition, the cells are easy to impregnate with metallic gold from acid solutions of gold chloride, and it was by this means that Langerhans (1868) first revealed them (see note 7). In more superficial strata, representing a later stage of involution, the cells round off or become more obtusely angular, and are still further reduced in size; the original processes, no longer tapered extensions of the perikaryon, are represented by short spikes or tenuous, fragile-looking threads.

No division stages or chromosome figures have been seen in Langerhans cells or in the products of their further degeneration.

8. The Regional Anatomy of the Pigmentary System

The epidermal melanocytes of adult mammals are members of one or other of two quite distinct sub-systems: that belonging to the hair follicles and responsible for the coloration of hairs, and that belonging to the superficial epidermis. The follicular system is an embryological derivative of the superficial, for melanocytes accompany the cells of the Malpighian system in the invagination or immigration of the superficial epithelium that establishes the primordia of hairs (cf. Zimmermann and Cornbleet, 1948; Chase and Smith, 1950; Rawles, 1953; Reynolds, unp.). In mice and rabbits, the melanocytes that stay behind in the superficial epidermis after the defection of those that enter the follicles remain at an insensibly low level of pigmentary activity (Reynolds, unp.); indeed, they are commonly supposed not to be there at all; but in human beings, guinea-pigs, cattle, and pigs, all more coarsely and sparsely haired, the superficial epidermis is thicker and the melanocytes may retain a high degree of pigmentary activity.

The two sub-systems are anatomically independent, because although the epithelial investment of the body is continuous, the melanocyte system is interrupted by a gap in that region of the hair follicles to which the sebaceous glands are attached. Inasmuch as the numbers of melanocytes in the follicles are not added to by later immigrations from the superficial epidermis or elsewhere, their loss entails their loss for good (Taylor, 1949; Chase and Smith, 1950) unless steps are taken to replace them surgically (Billingham and Medawar, 1953). Moreover, the pigmentary activities of the melanocytes of the two systems may be conspicuously different. In white human beings, for example, black hairs penetrate a very feebly pigmented skin; in pigs, white bristles may pierce a black skin; in red-and-black patched guinea-pigs, there is a zone transitional between red and black areas in which red hairs may be seen to issue from a black skin. Nevertheless, as their common origin might lead one to predict, members of the two systems are fully interchangeable. If the superficial epidermis of hair-bearing skin on the guinea-pig is removed in such a way as to leave the bases of the hairs intact, it will be formed anew from epithelium and melanocytes that have migrated upwards from the follicles.

Conversely, black melanocytes may be introduced into red or white follicles by direct inoculation of cells scraped from the under side of the superficial epidermis (Billingham and Medawar, 1953; Pepper, unp.). Apart from differences of colour—these, it will be seen, are of specific rank—it is therefore unlikely that the melanocytes of the two systems are anything more than topographical variants of the same cellular species. Indeed, with the same reservation still in mind, it could be argued that all the melanocytes of the epidermis, no matter what the region of the body in which they happen to reside, are embryologically homogeneous and fully interchangeable.

Melanocytes are not present in all epithelia of epidermal or ectodermal origin. They are, for example, absent from the corneal epithelium and, in some mammals, from the epithelium of the oral mucosa and the tongue.

The colour variants of skin and hair are the subject of a tremendous literature of genetical, embryological, ethnographical, and clinical research (see note 8), but our knowledge of the micro-anatomical background of colour variation is still rudimentary. Many of the colour differences to be found in a single animal are of purely contingent origin: they may be produced by differences of exposure to actinic radiation—sunburning is its literally photographic record—or, as in Himalayan rabbits and 'albino' guinea-pigs, in consequence of the fact that the points are generally cooler than the rest of the skin; localized depigmentation of the skin, 'leucoderma', is a minor hazard in industries that make use of anti-oxidants; pigmentation may vary from one time to another in a single individual in response to alterations in the output or balance of gonadal or adrenal hormones; and so on. It is some measure of our ignorance of the micro-anatomy of pigmentary variation that no description of the pigmentation that accompanies Addison's disease has yet improved upon that provided by its discoverer a century ago. There is just enough evidence to be fairly confident that melanocytes change neither their number, distribution, nor structure in response to treatments that enhance or subdue their pigmentary activity. It was at one time believed that dendritic processes actually developed *de novo* in response to actinic stimulation; all that it does, however, is to increase their melanin content and Dopa reactivity, so making them easier to see. The melanocytes of white human beings are no less arborescent than those of negroes; nor, it appears, are they less numerous (Billingham, 1949).

Superimposed upon differences of environmental origin, using that term in a wide sense, are the *innate* differences of pigmentary activity that distinguish the variously coloured areas of a patched or spotted animal. Thorough numerical studies of the cellular composition of the skins of parti-coloured—particularly red and black—guinea-pigs by Billingham and Medawar (1953) have failed to reveal any differences of structure, number, or distribution between the melanocytes of differently coloured areas. Choosing pairs of skin samples taken from symmetrically opposite areas, one red, the other black, these authors have recorded the following mean population densities of melanocytes per mm.^{2} of the basal layer of the epidermis in plane projection:

	Ear skin	*Trunk skin*
Red skin . . .	866±101	392±30
Black skin . . .	920±144	420±39

The standard errors show that variations between one animal and another are considerable, but the numbers of melanocytes in red and black skin vary in step with each other, and the two sets of estimates are highly correlated ($r = 0{\cdot}84$ for red and black ear skin and 0·86 for red and black skin from the trunk). The difference between red and black skins therefore appears to be a consequence *only* of differences between the pigmentary activities of the individual melanocytes within them. Inasmuch as Langerhans cells are visible in the white areas of recessively spotted guinea-pigs (Billingham, 1948) and mice (Reynolds, unp.), we must suppose that the entire superficial epidermis is populated by a web of melanocytes, the numbers of which vary from place to place without regard to differences of colour.

There is one respect in which the variously coloured areas of patched guinea-pigs, cattle, and sheep are not fully autonomous. Between any two such areas there is a transitional zone; for example, in red-and-black patched guinea-pigs, red skin bearing red hairs is separated from black skin bearing black hairs by a narrow band of black skin bearing red hairs. Indeed, if a black graft is transplanted into red skin, the latter is slowly but steadily encroached upon by black pigmentation spreading outwards from the graft. This new pigmentation is normally confined to the superficial epidermis; the hairs remain of their original colour. In the areas in which 'pigment spread' has occurred, black melanocytes are found to be present in the same number and distribution as the red melanocytes which were there beforehand; there is no intermixture of the two. Exactly analogous phenomena occur (very much more rapidly) at the margins between black and white or between red and white areas; and in the skins of cattle (Anderson, Billingham, Lampkin, and Medawar, 1951) and sheep (Hardy, Fraser, and Short, 1952) as well as guinea-pigs and the tails of mice (Reynolds, unp.).

Billingham and Medawar (1948*a*, 1950*a*) attribute pigment spread to the propagation from cell to cell of an *inherited* transformation of red (or white) into black (or red) melanocytes. It is the normal everyday activity of melanocytes to inoculate Malpighian cells with certain of their cytoplasmic ingredients. Malpighian cells are, however, so constituted as to be unable to support pigmentary activity, and deprived of an extraneous source, their pigmentation merely fades away. But at the dividing line between, for example, red and black skin, red and black melanocytes may unite by fusion of their branches in much the same way as a black melanocyte will fuse with another black, or one red with another (figs. 9 and 10). Billingham and Medawar argue that if some part of the cytoplasm of a black melanocyte enters a red neighbour across this cytoplasmic bridge, the pigmentary system of the latter may be permanently transformed; and a red melanocyte so transformed may in its turn transform another. Pigmentation therefore spreads in a manner closely analogous to an infection. A related phenomenon is believed to occur in consequence of the ingestion by melanophages of cytoplasmic matter from the melanocytes of newts (Niu and Twitty, 1950). So far as the guinea-pig is concerned, 'pigment spread' may be regarded as a minor developmental curiosity of considerable theoretical import; for if Billingham and Medawar's interpretation is correct, it implies that differences between the various melanocytes of a single individual are due to differences of cytoplasmic and not of nuclear properties; and this may be true, though much less conspicuously true, of all the inherited differences between the cells that comprise the progeny of a single zygote.

NOTES

1. The word *endothelium* seems to be used (*a*) as a special anatomical term to describe the cells of secondarily epithelial habit that line the inner walls of blood and lymph vessels, and (*b*) to distinguish an epithelium which bounds a concave surface from one that bounds a convex surface, where failure to do so would be confusing (e.g. the cornea). 'Mesothelium' (apparently a portmanteau word meaning 'mesodermal epithelium') may have been coined in the mistaken belief that the prefixes *epi-* and *endo-* are in some way indicative of embryological origins. However that may be, it is a neologism that might well be dispensed with.

2. The argument is not weakened by the fact that some fraction of the oxygen used by epidermal cells may be provided by diffusion from the exterior. Smelser and Ozanics (1952), studying the reversible minor defects of vision that follow the prolonged wearing of contact lenses, give good evidence that a certain necessary fraction of the oxygen supply of the cornea comes by diffusion from its outer surface (see also Billingham and Medawar, 1950*b*). The subject of the permeability of normal skin to gases has been reviewed by Calvery, Draize, and Lang (1946).

3. It is highly misleading to speak, as biologists are so inclined to do, of an 'innate predisposition' of skin to darken, crease, or thicken; for to say that skin which darkens upon exposure to sunlight has an innate predisposition to do so is merely a roundabout way of saying that it does indeed darken. The *difference* between the degrees of pigmentation of the two arms is caused by a *difference* between the conditions to which they have been exposed; and the number, distribution, and pigmentary potentialities of the melanocytes, being the same for both arms, can be regarded as parameters of the experimental situation. But when homologous areas of skin on two different individuals darken to different degrees after exposure to the same dosage of sunlight, then—other things being equal—it may be proper to speak of innate *differences* in their capacity to darken. Failure to observe these simple rules of formulation, and the habit of referring to 'innate properties', rather than to innate differences of property, has lead to widespread confusion in many branches of biological science.

4. The fact that thickened soles and palmar creases are, in any individual's lifetime, of developmental origin, though they could perfectly well be of functional origin, is a matter of some interest in evolutionary theory: cf. Medawar, 1954.

5. Melanophore, melanoblast, melanogenocyte, melanodendrocyte, pigmentary dendritic cell, chromatophore, stellate cell, clear cell, Langerhans cell (but see later), and *cellule amboceptrice* are among the synonymy of the cells of which *melanocyte* is now the agreed description (see the preface to *Pigment cell growth*, ed. M. Gordon,

N.Y., 1953). Notwithstanding this agreement, 'clear cell' may still be used to describe the perikaryon of a melanocyte in weakly pigmented skin, as it is seen n transverse section, and 'dendritic cell' (Becker, 1927) should be kept as a general term for melanocytes and their derivatives in superficial layers of the epidermis.

6. In certain areas of skin—e.g. the dorsum of the mouse's ear distally—the perikarya of many of the melanocytes lie entirely within the corium. See Holmes (1953).

7. Langerhans correctly supposed that the cells which he was the first to describe occupied an intermediate position in the epidermal strata. Billingham and Medawar for some time erroneously supposed that Langerhans cells were present in the basal layer, and others have followed them in this interpretation. The mistake is due to the fact that, after treatment with weakly acid solutions, the epidermis tends to separate from its substratum, not at the dermo-epidermal interface, but in a more superficial plane—so giving rise to the impression that Langerhans cells lie in the innermost tier of the epidermis.

8. Many of these lines of research are brought together from time to time in conferences on the 'Biology of Normal and Atypical Pigment Cell Growth', the proceedings of the first three of which are to be found in: *Sp. Publ. N.Y. Acad. Sci.*, **4**, 1948; *Zoologica*, **35**, part 1, 1950; *Pigment cell growth*, Academic Press, N.Y., 1953.

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Surface Forces in the Tracheal System of Insects

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SUMMARY

The mechanism by which the tracheal system becomes filled with air is reviewed. is concluded that the fluid contents are actively absorbed by the walls of the ystem. The air often enters from the atmosphere through the spiracles. In some sects the system fills while the insect is submerged in water or while the spiracles re closed. It is shown by means of simple models how the tanning of the lining of the rger tracheae or the secretion of wax over the walls will bring about the liberation of as from solution when the fluid is subjected to a very slight negative pressure.

The movements of fluid in the tracheole endings of insects are also reviewed. The emoval of fluid which takes place during activity, particularly under conditions of eficient oxygen supply, is not caused by secretory activity but by the physical forces roduced by the products of metabolism.

The evidence supports the view that the fluid in the tracheoles is a cell sap whose assage up the tracheole under the action of capillarity is opposed by the elasticity or welling pressure of the cytoplasmic sheath of the tracheoles. It is shown by means of simple gelatin model how osmotic changes in the surrounding fluid, acting by way f a cytoplasmic sheath, will bring about the absorption of such a cell sap.

A more exact model, illustrating the greater permeability of the inner wall of the racheole which the proposed mechanism requires, is provided by the anal papillae of nosquito larvae. These structures show the same adaptation to a saline medium as is ostulated in the tracheoles of mosquito larvae from salt water.

Alternative mechanisms are discussed. It is suggested that the capillary forces in the racheoles are probably small. The effects of probable contamination of the tracheole luid by an oily film (Beament) and the effects of changes in hydrogen-ion concentraion on interfacial tensions are illustrated by reference to further models.

INSECTS, for the most part, are of such a size that surface forces are great enough to move or to support the whole mass of the body. This is particularly evident in the adaptations to be seen in those insects which live in or n the surface of water (Brocher, 1909) and in the mechanisms of adhesion by neans of which terrestrial insects hold on to smooth solid surfaces (Gillett nd Wigglesworth, 1932).

Surface forces are manifest also in the tracheal system of insects, where aseous, solid, and liquid phases meet. They create problems during the nitial filling of the tracheal system with air at the time of hatching from the gg, and they are concerned in the movements of fluid or the maintenance of as in the endings of the tracheoles throughout the life of the insect. In both hese regards there are striking differences in different insects and sometimes, it would seem, between the different parts of the body in the same nsect.

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A good deal of fragmentary information about these matters has accumulated during the past 30 years. It is a field in which the phenomena readil lend themselves to interpretation in terms of simple mechanical models. Th object of this article is to bring together these scattered observations and t formulate the questions that are still unanswered.

The Initial filling of the Tracheal System with Gas

In most insects the tracheal system is open to the exterior through functional spiracles. In some aquatic or parasitic larval forms the spiracles ar concerned only in the moulting of the tracheal linings during ecdysis and th tracheal system is functionally closed. But when we consider the initial fillin of the tracheal system with gas at the time of hatching from the egg there is n real distinction between the 'open' and the 'closed' system.

In the closed system, of course, gas first makes its appearance while th insect is still immersed in fluid. That is so in *Corethra* (Krogh, 1911; v Frankenberg, 1915), in Chironomids (Pause, 1918), in Ceratopogonid (Keilin, 1924), in *Sciara* (Keister and Buck, 1949), in Odonata (Calvert, 1898 Tillyard, 1915). But even in insects in which the tracheal system is open, ga may appear throughout this system while the larva is still bathed in the flui contents of the egg. That is so in the blowflies *Calliphora* (Weismann, 1863 and *Lucilia*, and in the caterpillar *Sitotroga* (Sikes and Wigglesworth, 1931) Here the mechanism must be the same as in the closed system.

In many insects the surface of the larva dries while it is still inside the egg and the tracheal system fills, before hatching, from the outside air (as in th flea *Ceratophyllus* and the mealworm *Tenebrio* (Sikes and Wigglesworth, 1931) Often the larva at the time of hatching is enclosed in a cuticle which retains a layer of fluid beneath it so that air has access to the tracheae and filling occurs only when this 'embryonic cuticle' is shed soon after hatching (as in the bed-bug *Cimex* and the louse *Polyplax* (Sikes and Wigglesworth, 1931)).

But even where filling does not normally occur until the spiracles are in contact with the air, the system may sometimes fill, like the closed system, even when the egg or young larva is kept permanently submerged. That is so in the mealworm provided that the egg receives an adequate supply of oxygen (Sikes and Wigglesworth, 1931). Other insects, such as *Ceratophyllus* or *Aedes* (Wigglesworth, 1938*b*), have not this ability.

Disappearance of Fluid from the Tracheal System

It was made clear by the work of Weismann (1863), Keilin (1924), and Davies (1927) that the fluid in the tracheal system is absorbed into the tissues. In the case of the open system this process has been described in detail in *Aedes aegypti* (Wigglesworth, 1938*b*). When the posterior spiracles of the *Aedes* larva come into contact with the atmosphere at the water surface, air enters almost at once and extends along one tracheal trunk and then the other as the fluid is absorbed. The system is completely filled in 15–30 minutes.

This absorption is almost certainly the result of active transfer or secretion y the cells of the tracheal system. The evidence is as follows:

(i) It does not take place in the absence of an adequate supply of oxygen. his was shown in the filling of the system of *Tenebrio* during submergence ikes and Wigglesworth, 1931). It was proved in detail by Keister and Buck 949) who showed that in the larva of *Sciara* the system (a closed system) oes not fill with gas if the oxygen concentration falls much below 0·3 per cent.

(ii) It is inhibited by narcosis. Larvae of *Aedes* narcotized with chloroform ill rest with the spiracles open at the water surface but no air enters. Even fter recovery from the narcotic there is a further delay of 10 minutes or so efore filling begins (Wigglesworth, 1938*b*). These observations suggest not nly that absorption is a secretory phenomenon but that the nervous system, cting perhaps through some hormonal mechanism, is concerned in starting he process. Filling is also inhibited by low temperature (0° C.) (Keister and uck, 1949).

(iii) Only for a limited period after hatching is absorption possible. Subnergence of newly hatched larvae of the flea *Ceratophyllus* for several days revents the system from filling with gas when the spiracles are later exposed o the air (Sikes and Wigglesworth, 1931). In *Aedes* larvae, filling of the system s somewhat delayed after submergence for 2 days; after 3–4 days filling is very low and usually incomplete (Wigglesworth, 1938*b*). That is so despite the act that the tracheal endings remain permeable to the tracheal liquid throughut the life of the insect. In the later larval stages the period during which bsorption can be effected is much shorter: second-stage larvae prevented rom coming to the surface for half an hour only, are unable to fill their racheal system (Wigglesworth, 1938*b*).

Nature of Tracheal Liquid and the Site of Absorption

It was earlier suggested that the fluid in the tracheal system in the egg might be an ultrafiltrate from the body fluids resulting from the expansion of he tracheal lumen during development, and that therefore it should be capable of reabsorption by osmosis (Sikes and Wigglesworth, 1931). This, however, is an improbable mechanism. In the embryo, the tracheal fluid is continuous with that in the amniotic cavity, which contains proteins and salts (Sikes and Wigglesworth, 1931). The same process occurs at each moult and here the tracheal fluid is continuous with the moulting fluid below the cuticle and presumably has the same composition. The moulting fluid in *Rhodnius* is a neutral fluid, apparently free from chloride, with proteins in solution (Wigglesworth, 1933*c*). In the *Cecropia* silk moth (Passonneau and Williams, 1950) it contains sodium and potassium as well as the breakdown products of protein and chitin. Such a fluid could readily be absorbed by an active secretory process, though not by simple osmosis.

The moulting fluid is absorbed, shortly before moulting, through the general surface of the cuticle (Wigglesworth, 1933*c*). The absorption of the tracheal fluid at the time of hatching and during each moult is doubtless comparable process and probably takes place throughout the system. This belief is supported by certain observations on *Aedes* in which the main tracheal trunks are connected in each segment by a transverse trachea from which no tracheoles are given off. During filling it is quite common to see columns of air enter these transverse branches from both ends simultaneously.

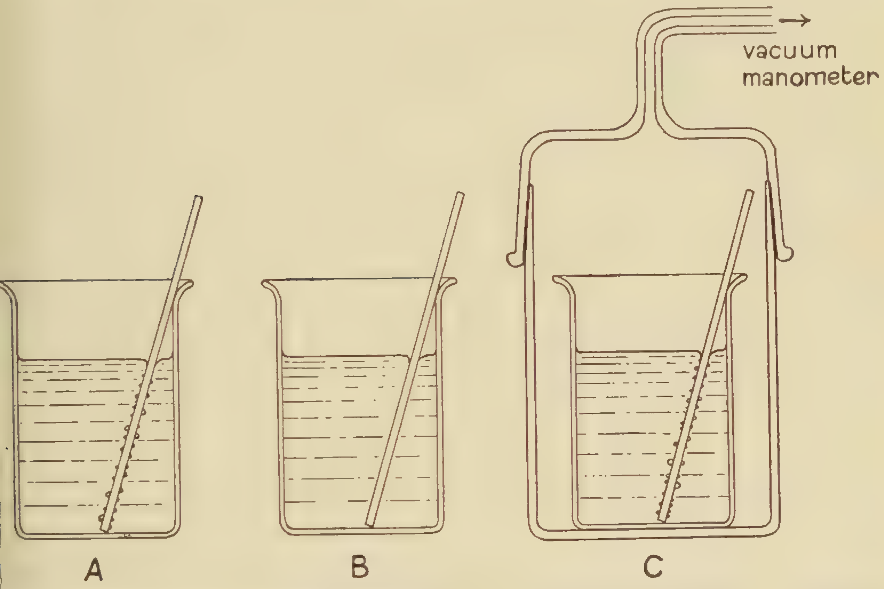
FILLING OF THE CLOSED OR SUBMERGED TRACHEAL SYSTEM: LIBERATION OF DISSOLVED GASES

There can be little doubt that the removal of fluid from the tracheal system and its replacement by air is brought about by active absorption. (In their discussion of this problem Keister and Buck (1949) attribute to the present writer the belief that an active secretion of gas is responsible; but in the paper in question (Wigglesworth, 1938*b*) it is concluded that 'the fluid present in the tracheal system is removed . . . by an active absorption'.) It is unlikely that there is any fundamental difference between the filling of the open system from the atmosphere and the filling of the closed system from gases in solution. For, as we have seen, certain insects such as *Tenebrio*, which normally take in air directly from the atmosphere, are capable of filling when submerged.

The chief problem here is the force necessary to rupture a continuous column of liquid (Keilin, 1924). This force may be very great. The experiments of Dixon (1914) have shown that even with air in solution the cohesion of water amounts to about 200 atmospheres' pressure. But in order to obtain this high figure for adhesion and cohesion of water very special precautions had to be taken to remove all grease from the apparatus and to ensure that every part was thoroughly wetted. If this was not done, free gas appeared when the pressure was only slightly reduced; and Dixon admits that air bubbles are common enough in the conducting tissues of the stems of plants although the negative tension (due to the transpiration force) to which the sap is exposed is only between 5 and 20 atmospheres.

That is so in spite of the fact that the conducting tubes of plants imbibe water and therefore must exert a very strong adhesive power on the sap. The tracheal intima of insects, on the other hand, has a very low adhesion to water. So far as the general surface of the body is concerned, the cuticle becomes completely non-wettable by water as the result of the deposition of a crystalline layer of wax immediately before moulting occurs (Wigglesworth, 1948). The wetting properties of the tracheae are such that it seems highly probable that the larger branches likewise have a waxy lining. If the deposition of this wax (or the quinone tanning of the cuticle, which will likewise reduce the adhesion to water) coincides with the development of a small negative pressure within the tracheal system, due to a process of active absorption, it is not surprising that gas should be liberated from solution in the larger tracheae. Once a bubble of gas has formed it will grow rapidly in size as the tracheal fluid is absorbed and dissolved gases diffuse into the expanding bubble.

This process can be illustrated by means of a simple model experiment, a odification of one previously described (Sikes and Wigglesworth, 1931). A aker thoroughly freed from grease with chromic acid is partly filled with ater from the cold tap—water, that is, which is supersaturated with air when ought to atmospheric pressure. No bubbles of gas are liberated. A slide vered with paraffin wax is then placed in the beaker, and bubbles of gas are on liberated from the supersaturated water, but only on the waxed surface fig. 1, A). If the waxed slide is placed in a similar beaker containing distilled vater that has been exposed to the air for some days, no bubbles of gas are et free upon either surface (fig. 1, B). But if now the pressure is lowered by a uarter of an atmosphere, minute bubbles appear upon the waxed surface vithin a few minutes. At a negative pressure of half an atmosphere the ubbles appear almost at once and grow rapidly in size, but no gas is set free n contact with the clean glass (fig. 1, C).



IG. 1. Experiments to illustrate the effect of wax in causing the liberation of gas from solution. (Explanation in text.)

We are still faced with the problem of why the tracheal system in some nsects (*Calliphora* (Weismann, 1863), *Lucilia*, *Sitotroga*, *Tenebrio* (Sikes and Vigglesworth, 1931), *Corethra* (v. Frankenberg, 1915), *Ceratopogon* and *Chiroomus* (Keilin, 1924), *Sciara* (Keister and Buck, 1949), Odonata (Tillyard, 916)) will fill with gases from solution, whereas in others (*Cimex*, *Polyplax*, *eratophyllus* (Sikes and Wigglesworth, 1931), *Aedes* (Wigglesworth, 1938*b*)) t will fill only from the atmosphere. The difference may lie in the nature and vetting properties of the tracheal lining or in the intensity of the absorptive ctivity of the cells.

It may be recalled that Weismann (1863), who observed the filling of th tracheal system in *Calliphora* with gases from solution, explained the proces as follows: the trachea when first formed has only a potential lumen. Then cuticle is laid down, the lumen expands, and fluid diffuses into it from th tissues. He supposed that at a certain stage the cuticle of the larger trunk becomes less permeable to fluid; but the lumen goes on enlarging and henc the space must be filled with gas diffusing from the tissue fluids. Finally Weismann supposed that the residual fluid is absorbed into the tissues throug the finest tracheal tubes. Actually the lumen seems to be fully formed befor the appearance of gas, but the possibility of negative pressure being produce within the tracheal system of some insects in this way is worth bearing in mind. (Sikes and Wigglesworth (1931) invoked a somewhat similar process to explain the appearance within the tracheae of an ultra-filtrate from the tissue that could be reabsorbed by osmosis. This idea has since been abandoned in favour of absorption by a secretory process as described above.)

As to the nature of the gas which first appears in the closed tracheal system, a subject which has provoked much discussion, there is no reason to suppose that this will be constant. As already pointed out (Sikes and Wigglesworth, 1931) its composition will depend solely upon the partial pressures and the invasion coefficients of the various gases in solution in the tissue fluids at the moment when filling begins.

Extent of Air in the Tracheoles

In the past there has been some doubt as to whether the endings of the tracheoles during life contain liquid or gas. Observations on the living insect have shown that there is much variation in different insects and in different tissues in the same insect. In some tracheoles the column of air ends abruptly, long before the tracheole comes to an end. That is so in the tracheoles in the head and anal papillae of mosquito larvae (Wigglesworth, 1930), the endings on the gut of *Aeschna* (Wigglesworth, 1930) and *Tenebrio* larvae (Wigglesworth, 1931), in many parts of the body in *Blattella* (Wigglesworth, 1931; Bult, 1939), many of the endings in the abdomen of the flea (Wigglesworth, 1931, 1935), the adult mosquito, and the bed-bug *Cimex* (Wigglesworth, 1931). In other endings the tracheoles contain gas as far as they can be resolved with the light microscope. That is so in the leg muscles of the flea, in the muscles of the body wall of an active mealworm larva, and apparently in all the organs of *Pediculus* (Wigglesworth, 1931). Whether those branches which contain air as far as they can be resolved do in fact contain gas up to their extremities is not certainly known, but it seems likely that this is so.

Measurements made recently on the tracheoles of the epidermis in *Rhodnius* (Wigglesworth, 1953) show that these spring from the endings of small tracheae usually 1·5–2μ in diameter. At their commencement their internal diameter varies from 0·9 to 0·6μ. They run a course of 200–300μ (with a single nucleus lying at about 75μ from their point of origin), diminishing very gradually in diameter and sometimes giving off a single branch, to end at an parent diameter of about 0·2μ or less. Electron microscope studies have own that the tracheoles, in which no spiral filament is visible with the light icroscope, do in fact have their walls thrown into annular or spiral folds like e larger tracheae and that they end abruptly in rounded form at a diameter 0·5–0·2μ (Richards and Korda, 1950).

Movements of Fluid in the Tracheoles: Osmotic Pressure

In those forms in which the tracheoles contain liquid the extent to which it ses up the tubes is not constant but varies with the metabolism in the tissues: ie fluid is absorbed and gas extends farther along the tracheoles after active uscular contraction, or in the case of tracheoles on the surface of the gut, at ie height of digestion. Removal of fluid is most active during asphyxia ausing violent muscular activity in the absence of oxygen (Wigglesworth, 930, 1931, 1935). A similar removal of fluid is brought about if the tracheal ndings are exposed to hypertonic solutions of sodium chloride, potassium ictate, &c. One per cent. sodium chloride or 2 per cent. potassium lactate vill cause complete removal of fluid just like asphyxia.

It was therefore inferred that the fluid was absorbed by osmosis into the lood; that the capillarity of the tracheole was in equilibrium with the osmotic ressure of the blood; and that when the osmotic pressure was increased ocally by the liberation of unoxidized metabolites into the blood, water was bsorbed from the tracheal endings so that the meniscus entered finer and ner tubes until a new equilibrium was established. As the metabolites were xidized or removed the local osmotic pressure of the blood was believed to all and the fluid therefore rose once more in the tracheoles.

Assuming that the fluid in the tracheoles is pure water with a surface ension equal to that of distilled water, that it wets the wall of the tracheole completely, with an angle of contact of 0°, and that the barrier between the racheole lumen and the haemolymph behaves as a simple perfect semiermeable membrane, it was calculated that the forces in question would just bout balance (Wigglesworth, 1930).

These, however, are very large assumptions and probably no one of them s justified. Indeed, the sole purpose of the calculation was to show 'that the theory put forward was not unreasonable on physical grounds'.

In discussing above the initial filling of the tracheal system with air we came to the conclusion that the fluid in the tracheae is of the same nature as the amniotic fluid or moulting fluid: a dilute lymph with which the cells are in osmotic equilibrium. It seems more probable that the fluid in the tracheoles at a later stage in development should also be of this character, than that they should contain a fluid that would be virtually distilled water.

The first indication of something of the kind was given by observations on mosquito larvae that had become adapted to salt water. The larva of *Aedes aegypti* reared in fresh water has an osmotic pressure in the blood equivalent to 0·75–0·9 per cent. sodium chloride. If it is acclimatized to dilute sea water (equivalent to 1·5 per cent. NaCl) the osmotic pressure of the blood rises to the equivalent of 1·5 per cent. NaCl, and yet *the fluid in the tracheoles of th resting larva rises to its accustomed level and is removed in the usual way upo asphyxiation.* Clearly there cannot be a simple balance between osmotic pressure in the blood and capillarity (Wigglesworth, 1938*a*).

COLLOID OSMOTIC PRESSURE OR SWELLING PRESSURE AND THE MOVEMENT O FLUID IN THE TRACHEOLES

In the original discussion (Wigglesworth, 1930) the wall of the tracheol was treated as a simple semi-permeable membrane. But it consists in fact o a sleeve of cytoplasm surrounding the tracheole wall. We are faced with th question whether osmotic changes in the haemolymph bathing such a system could bring about the removal from the lumen of the tubes of a fluid with which the cytoplasm is in osmotic equilibrium.

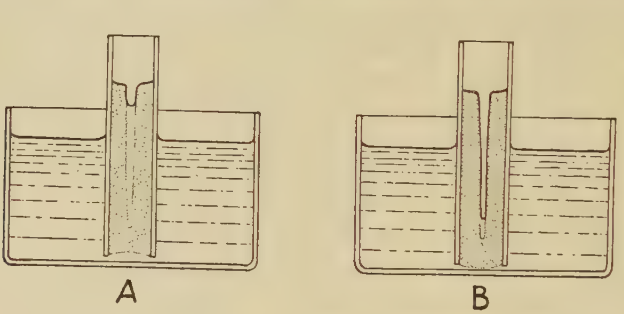


FIG. 2. Gelatin model of tracheole. (Explanation in text.)

We may consider a simple model prepared as follows (fig. 2). A glass tube of 0·7 cm. internal diameter, open at both ends, is filled with 10 per cent. gelatin in 50 per cent. Ringer's solution (Ringer's solution diluted with an equal volume of water). Before the gelatin has set the end of a capillary pipette is inserted into the tube so that its end comes to within about 3 mm. from the lower surface of the gelatin. When the gelatin has set the pipette is withdrawn, the cavity (which represents the lumen of the tracheole) is filled with 50 per cent. Ringer and the tube is stood upright in the same solution and left overnight for equilibrium to become established. The tube is then stood upright in 100 per cent. Ringer's solution; and it is found that the solution from the lumen is absorbed. In one experiment the column of fluid in the lumen was reduced from 13·5 mm. to 2·5 mm. during 24 hours. The capillary lumen tapered from 1·5 to 0·75 mm., so that about 90 per cent. of the fluid had been absorbed. In other experiments, the fluid was sometimes completely replaced by air.

Clearly in this model the increased concentration of salt has increased the swelling pressure of the gelatin which has then absorbed the more dilute solution from the lumen; a process which could be described in terms of the Donnan equilibrium combined perhaps with specific effects of the ions present in inducing swelling. The model serves the purpose of showing how ianges in osmotic pressure in the haemolymph might act indirectly through ie swelling pressure of the cytoplasmic sheath of the tracheole in causing a mporary absorption of a saline solution in the lumen (Wigglesworth, 1938*b*).

But the wall of the tracheole is a much more complex structure than this. he cytoplasmic sheath is bounded on the outside by a cell membrane parating it from the haemolymph, and on the inside by the modified cell iembrane which is the wall of the tracheole. The osmotic pressure of the aemolymph will operate through the outer cell wall upon this cytoplasmic ieath; and the capillarity of the tracheole will operate against the water bind-ig power, 'swelling pressure' or 'colloid osmotic pressure' of the cytoplasm. t is possible therefore to conceive a mechanism by which an increase in smotic pressure in the haemolymph will cause a temporary shrinkage of the ytoplasmic sheath. The framework or gel of this sheath will thereby be com-ressed. It will tend to expand again by reason of its elasticity (or of its col-oid osmotic pressure). If we suppose that the inner wall of the sheath, the racheole lining, is more permeable than the outer cell membrane, then fluid vill be absorbed from the lumen of the tracheole.

DIFFERENTIAL PERMEABILITY IN THE ANAL PAPILLAE OF MOSQUITO LARVAE

The crux of this mechanism lies in the conception of a differential per-neability between the inner and outer walls of the tracheole. Is such a struc-ure at all probable? It is scarcely feasible to construct a model of the same lelicacy as a tracheole wall having these properties; but it so happens that an xact model exists ready-made in biological material.

This is provided by the anal papillae of the mosquito larva *Aedes aegypti* fig. 3, A) (Wigglesworth, 1933*a*). These papillae are covered by a thin cuticle aid down by rather large cells separated from the haemolymph within the papilla by a cell membrane. It is suggested that the cuticle represents the inner wall bounding the lumen of the tracheole, the cells represent the cytoplasmic sheath, and the cell membrane represents the membrane which separates the cytoplasmic sheath from the haemolymph.

If the tip of the papilla is cut off and immersed in tap water so that this can reach the inner membrane, the cells swell by osmosis (fig. 3, B). If a similar isolated papilla is immersed in hypertonic saline (1 per cent. NaCl) the cells gradually shrink (fig. 3, C). Clearly water passes through the bounding membranes of the cells more readily than the salt.

On the other hand, if the intact larva is immersed in 1 per cent. NaCl or Ringer's solution the cells rapidly swell up to an enormous extent (fig. 3, D). If the larva is quickly returned to tap water the cells contract again (fig. 3, E). But if the larva is left in the hypertonic salt solution the cells are permanently injured and the cytoplasm dispersed (fig. 3, F). Clearly the salt enters the cells fairly readily through the outer membrane of the papillae but fails to pass so readily through the inner membrane; the cells thus become hypertonic to the haemolymph from which they absorb water and swell.

These differences in permeability between the outer and inner membranes of the anal papilla are precisely those which are postulated in the tracheoles. Since the lining membrane of the tracheoles is morphologically equivalent to the outer membrane of the anal papilla it is not unreasonable to suggest that it may have the same permeability characteristics.

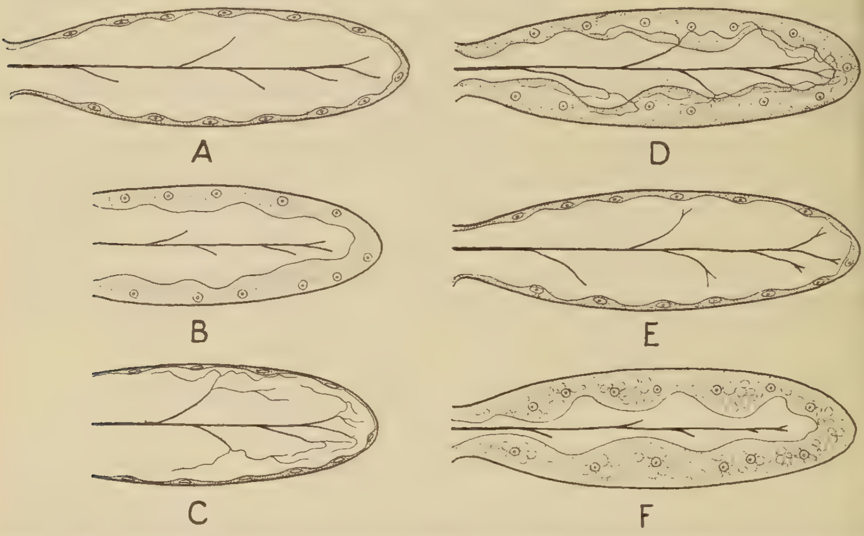


Fig. 3. Anal papilla of mosquito larva as a model to illustrate the permeability properties of the tracheoles. A, normal papilla of *Aedes aegypti*. B, tip of papilla cut off and immersed in tap water. C, tip of papilla cut off and immersed in 1 per cent. NaCl. D, papilla of intact larva immersed in 1 per cent. NaCl. E, the same restored to tap water. F, the same after prolonged immersion in 1 per cent. NaCl. The figures also show the changes taking place in the extent of air in the tracheoles.

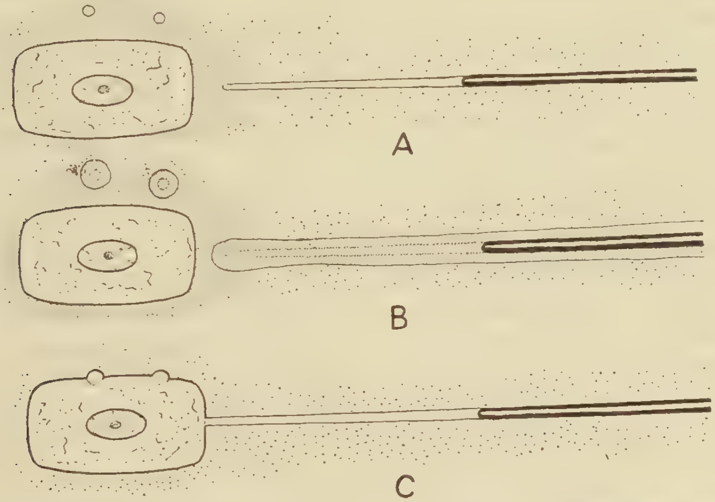
If the larva of *Aedes aegypti* is adapted to live in 1 per cent. NaCl, equilibrium becomes established between the cells of the papillae and the haemolymph, and the cells no longer swell when the larva is immersed in this solution (Wigglesworth, 1933*a*). That is what is believed to happen in the tracheoles of larvae similarly adapted (Wigglesworth, 1938*a*).

ALTERNATIVE MECHANISMS FOR THE CONTROL OF MOVEMENT OF THE TRACHEOLE FLUID

The mechanism outlined above is an elaboration of that put forward to explain the adaptation that occurs in the tracheoles of mosquito larvae reared in salt water (Wigglesworth, 1938*a*). Another mechanism, similar in principle though differing in detail was put forward by Bult (1939). These various mechanisms are illustrated diagramatically in fig. 4.

Fig. 4, A shows the original simple mechanism. The tracheole contains water and is bounded by a simple semi-permeable membrane. A rise in osmotic pressure, due to the activity of adjacent cells or the addition of a pertonic solution, draws water from the tracheole until a new balance is tablished between osmotic pressure and capillarity.

Fig. 4, B shows the mechanism described above. The tracheole is surrounded a cytoplasmic sheath separated from the haemolymph (or from the cyto- asm in the case of intracellular tracheoles) by a semi-permeable membrane. rise in osmotic pressure in the haemolymph causes shrinkage of the cyto- asmic sheath and thereby increases the forces of imbibition in that sheath.



IG. 4. Diagrams to illustrate the various schemes suggested to explain the movement of luid in the tracheoles. A, Wigglesworth (1930); B, Wigglesworth, 1938 and present paper; C, Bult (1939).

Since the lining membrane of the tracheole is more permeable than the outer membrane of the cytoplasmic sheath, the cytoplasm tends to swell again by absorbing the cell sap which has been drawn up the tracheole.

Fig. 4, C shows the mechanism proposed by Bult (1939). The tracheole is pictured as coming directly into contact with the cell, and as being separated from it by a membrane of greater permeability than the wall of the tracheole elsewhere or the surface membrane of the cell itself. The tracheole contains a cell sap which is drawn up the tube by capillarity until it is arrested by the resistance of the cell to collapse—that is, by the force of imbibition. A rise in osmotic pressure outside the cell will extract water from the cell. The elastic recoil of the cell opposes this shrinkage, and since the membrane separating the cell from the tracheole is the more permeable, the fluid from the tracheole will be absorbed into the cell. (Bult suggests that in normal circumstances the increased imbibition by the cell is not the result of increased osmotic pressure outside but is caused directly by oxygen want. This matter will be discussed later.)

In principle this mechanism proposed by Bult is the same as that illustrated in fig. 4, B, except that the cytoplasm of the cell takes the place of the cytoplasmic sheath of the tracheole. This makes it necessary to postulate a specially permeable region of the tracheole in contact with the cell. At presen there is no evidence for such an arrangement. Indeed, electron microscope studies show the tracheoles ending blindly without any change in the wall (Richards and Korda, 1950). But when the tracheoles penetrate within the cells (see fig. 3, D) there is little difference between schemes B and C (fig. 4).

VITAL ACTIVITY AND PHYSICAL FORCES IN THE TRACHEOLE ENDINGS

Throughout this discussion of the movements of fluid in the endings of the tracheoles it has been tacitly assumed that the wall of the tracheole plays a purely passive role, being subject to the physical forces exerted upon it. But we have seen that during the initial filling of the tracheal system with air at hatching or at moulting the fluid is removed by an active process of secretion. The question arises whether secretory activity may be responsible for the movements of fluid in the functional tracheal system.

It is quite certain that the tracheole is by no means a lifeless structure. It has recently been shown (Wigglesworth, 1953) that the tracheole endings will actively migrate considerable distances (perhaps 300–500μ) towards a region of deficient oxygenation, drawing the tracheae after them. There is no reason therefore why they should not have retained their secretory function—although, as pointed out above, removal of fluid from the tracheal system of the newly moulted insect is not possible if filling is delayed.

There can be little doubt that when the tracheal endings are exposed to hypertonic solutions the extraction of fluid is a passive osmotic phenomenon. This can be brought about by 1 per cent. NaCl, by Ringer's solution osmotically equivalent to 0·9 per cent. NaCl, or by 2 per cent. potassium lactate, which is about equivalent to 1 per cent. NaCl. When unneutralized lactic acid is used a rapid extension of air is followed by an equally rapid retreat. This was attributed to a breakdown in permeability. The same thing happens when other poisons such as hydrogen cyanide, ammonia, sulphur dioxide, or chlorine are employed, and may perhaps be interpreted as showing that we are concerned with the permeability of a membrane which is an inherent part of a living cell rather than some inert cuticle (Wigglesworth, 1930).

It was originally considered that the removal of fluid which follows asphyxiation is also due to the liberation of osmotically active substances by the contracting muscles. The reasons for this conclusion were as follows:

(i) Actual activity, particularly muscular activity, is necessary. In mosquito larvae submersion is much more effective than exposure to hydrogen because it causes much greater muscular activity. Pure hydrogen is more effective than pure carbon dioxide—because the larva is quickly narcotized in carbon dioxide and ceases to move.

(ii) Muscular activity may sometimes over-ride the effects of oxygen supply. If the mosquito larva is exposed in hydrogen the movement of fluid is arrested when the larva comes to rest. If it is then exposed to oxygen it immediately ecomes active and there may be an extension of air in the tracheoles before he larva becomes quiet and the air begins to retreat again.

(iii) Muscular activity in one part of the body may affect the tracheoles elsewhere. In the larva of *Aedes* muscular contractions in the head and trunk ause a delayed removal of fluid from the tracheoles in the anal papillae. And f a larva is asphyxiated by submersion until its tracheoles are filled with air, ts haemolymph will cause the removal of fluid from the tracheoles of a resting nsect (Wigglesworth, 1930).

All these observations suggest that the movement of fluid is caused, not by naerobiosis as such, but by the products of muscular or secretory activity iberated from the cells, particularly when the oxygen supply is deficient. Or, lternatively, that increased imbibition of water by the cells results in increased osmotic pressure outside them.

In opposition to this view Bult (1939) has suggested that the increased mbibition by the cells, which according to his view is responsible for absorbing fluid through the permeable wall of the tracheole in contact with the cell fig. 4, c), is the direct result of a low oxidation-reduction potential in the cytoplasm. He suggests that 'it is not at all improbable that . . . the redox-potential might affect the swelling of cell proteins'. He supposes that under conditions of reduced oxygen tension the cell proteins imbibe water and the cells swell. And since the surface of the cell is pictured as being less permeable than the membrane between the cell and the tracheole (fig. 4, c), fluid is absorbed preferentially from the lumen of the tracheole.

We have seen that the relation between cell and tracheole as described by Bult is improbable. But the fact remains that protoplasmic imbibition ncreased by the mechanism he suggests might well result in an increase in osmotic pressure outside the cell and so a removal of fluid from the tracheoles as outlined above. Or, if the effects of reduced oxygen tension were pictured as occurring actually within the cytoplasmic sheath of the tracheoles, then they would exert a direct effect on the fluid in the tracheole as already described.

Other indirect effects of this kind, notably the effect of acidity following oxygen want, in causing increased protoplasmic imbibition were briefly considered in the original paper (Wigglesworth, 1930). The possibility of such changes cannot be disregarded.

THE EFFECTS OF KNOWN CHANGES IN THE PARTIAL PRESSURE OF OXYGEN

Another phenomenon that must be taken into account is the changed equilibrium between osmotic pressure or imbibition and capillarity which develops when the partial pressure of oxygen is changed. This is well seen in the abdominal tracheoles of the flea (Wigglesworth, 1935). There are fluctuations in the level of fluid as the flea struggles or remains at rest; at a raised temperature of 35° C. the fluid is continually moving up or down the tubes (fig. 6, A and B); but the average level in the flea at rest remains tolerably constant while the insect is in air (fig. 5, air). But if the oxygen content of

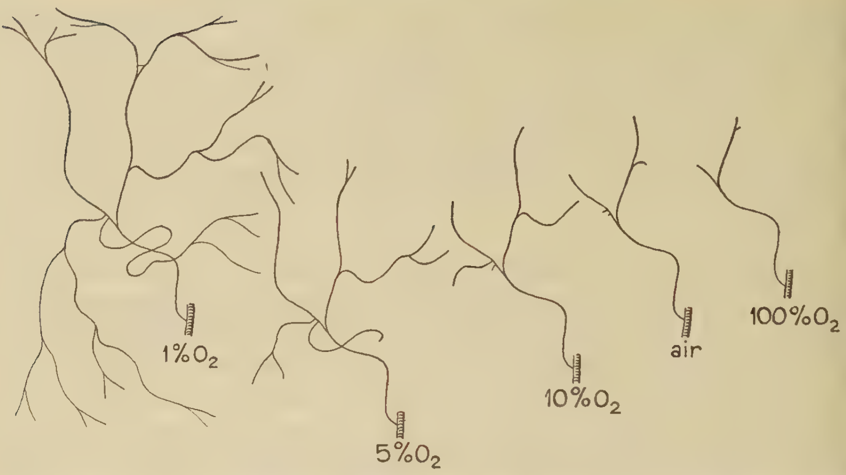


FIG. 5. Extent of air in tracheoles of flea at rest when exposed to different gas mixtures. Composite figure based on several experiments.

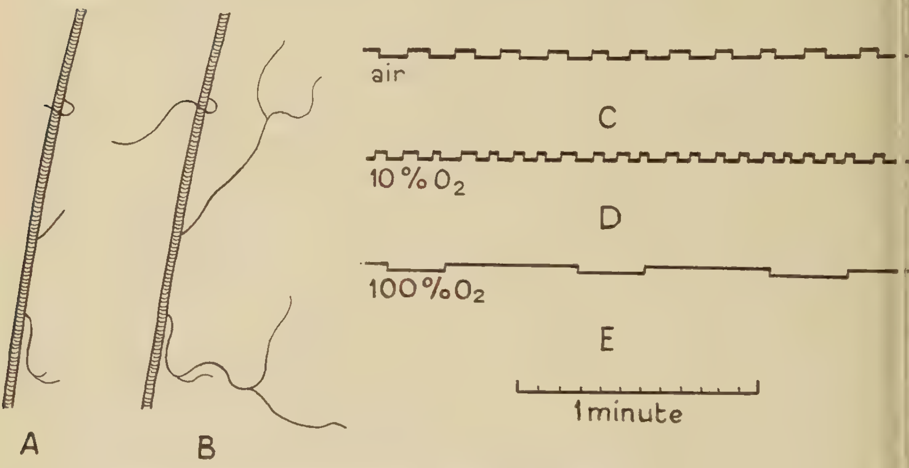


FIG. 6. A, tracheoles arising from trachea in abdomen of flea at rest in air at 35° C. B, the same, 5 seconds after commencement of struggling. C, opening and closing of abdominal spiracles of flea at rest in air. The upper lines representing the closed periods, the lower lines the open periods. D, the same in 10 per cent. oxygen. E, the same in pure oxygen.

the atmosphere is reduced to 10 per cent., the average position during rest is farther along the tubes (fig. 5, 10% O_2). In 5 per cent. oxygen still more fluid is extracted from the ends of the tracheoles (fig. 5, 5% O_2), whereas in 1 per cent. oxygen the tubes contain gas as far as they can be resolved (fig. 5, 1% O_2). On the other hand, if the flea is exposed to pure oxygen the average level of the fluid in the tracheoles during rest rises a little higher than it does in air (fig. 5, 100% O_2).

Clearly there is a different equilibrium in the metabolism of the tissues at ch partial pressure of oxygen, and one may use this material to provide idence that there is indeed an increased accumulation of acid metabolites hen the partial pressure of oxygen is reduced.

The spiracles in the flea, at rest in air, open and close in a fairly regular ythm as shown in fig. 6, C. The duration of the closed period is determined) by the accumulation of acid metabolites due to oxygen want, and (*b*) by the cumulation of carbon dioxide. The duration of the open period is deterined by the time taken for the carbon dioxide which has accumulated in the ody during the closed period to diffuse out. The duration of the open period therefore largely determined by the duration of the closed period which recedes it. That is why the open period is so greatly prolonged in pure xygen (fig. 6, E) and so much shortened in 5 per cent. oxygen (fig. 6, D). In ther words, when the insect is in pure oxygen, opening is induced by a large mount of CO_2, whereas when the insect is in 5 per cent. oxygen opening is duced by a very small amount of CO_2. It follows, therefore, that the mean vel of accumulation of acid metabolites must be greater at low partial presures of oxygen than at high—in agreement with the mean level of fluid in the racheoles. It is therefore reasonable to ascribe the removal of fluid from the racheoles to these same metabolites.

CAPILLARITY OF THE TRACHEOLES

When it was assumed that the osmotic pressure of the tissue fluids was actng in direct opposition to the capillarity of the tracheoles, we had to consider pposing forces of the order of 10 atmospheres. That would indeed be the apillary force in the tracheoles if they contained pure water and had walls hat were completely wettable.

According to the arguments set out in this paper we are in fact dealing with orces (colloid osmotic pressure or elasticity in the cytoplasmic sheath of the racheoles) which are probably only a fraction of an atmosphere.

That presents no difficulty. The tracheole wall is readily wetted by oils kerosene, &c.) which rapidly fill the system completely. The surface is ipophilic as well as hydrophilic; the angle of contact with water is therefore nlikely to be as small as 0°. Moreover, as Dr. J. W. L. Beament has pointed ut to me, if the fluid were covered with a film of lipide material its surface ension would be greatly reduced. The cuticle of the cockroach is covered vith a mobile grease (Ramsay, 1935) consisting of a wax in a volatile solvent Beament, 1951) and it seems very probable that this material will spread hroughout the lining of the tracheal system. In some unpublished observaions to which I am kindly permitted to refer, Dr. Beament noted that in certain clean glass capillary, distilled water rose to a height of 23 cm., vhereas when cockroach grease was added to the distilled water it rose only o 10 cm.

It may well be, therefore, that quite small forces are at work in moving the luid up and down the tracheoles. And it is possible that specific and local ariations in the wetting properties of the tracheole lining may account for the specific and local differences in the extent of air into the tracheoles alread described.

There remains the possibility that the surface tension of the fluid in contact with the walls of the tracheoles may vary under different physiologica conditions. There are large changes in tension at an oil-water interface followin slight changes in hydrogen-ion concentration. If the oil phase contains carboxy groups, either free or combined with glycerol, a fall in pH (such as might b expected to follow muscular contraction in the absence of oxygen) causes rise in interfacial tension (Hartridge and Peters, 1922). This would tend t make fluid rise in the tracheoles, which is the opposite to what is observed But if the oil phase contains long-chain amines, a fall in pH within th physiological range of pH 5 to pH 9 causes a fall in interfacial tension (Peters 1931).

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The Atrial Nervous System of Amphioxus (*Branchiostoma*)

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With two plates (figs. 1 and 2)

SUMMARY

This paper is concerned with the system of neurons which lies in the walls of the atrium of *Branchiostoma*.

The system is found on the parietal and ventral walls of the atrium, as well as round the gut, and it is therefore thought preferable to speak of it as the atrial nervous system rather than the autonomic (enteric) system (Boeke).

The neurons are shown to lie in or immediately below the atrial epithelium, which is of ectodermal origin.

Special attention is given to the innervation of the gut and its diverticulum, of the atrio-coelomic funnels, and of the atrioporal region.

Evidence as to the effector and receptor elements of the system is discussed. It has central connexions, and it is suggested that its activities play an important part in the animal's behaviour.

'AMPHIOXUS has been quite a speciality of the Journal', wrote Bourne in 1919, and it was in the pages of the Q.J.M.S. that there appeared a work which has had a considerable influence on interpretations of the peripheral nervous system of this animal (Boeke, 1935*a*). The nature of this influence is shown in Grassé's *Traité de Zoologie:* 'L'éxistence d'un système sympathique a été démontrée par Boeke d'une manière irréfutable' (Drach, 1948).

Boeke's material consisted of silver-impregnated sections of the gut of amphioxus, and he described the neurons that he found in these; he suggested that their location, cytology, and interrelations were such as to justify structural and functional homology with the visceral autonomic system of higher chordates.

The viscera of amphioxus lie in a special cavity, the atrium, and workers before Boeke had found neurons not only on the visceral side of the atrium, against the gill bars and gut, but also on its parietal side, against the somatic musculature. These were more than once called 'sympathetic nerves', but before Boeke's time the homology with the craniate autonomic did not gain general acceptance, perhaps because Franz, who wrote accurately and at length on amphioxus, was not convinced of their existence. It is convenient to use the phrase *atrial nervous system* to refer to the neurons on the visceral and parietal borders of the atrium, for they are connected with each other.

[Quarterly Journal of Microscopical Science, Vol. 94, part 4, pp. 523–535, December 1953.]

The anatomy of the connexions between the atrial and central nervou systems has been established by several investigators (literature surveyed b Franz, 1927). Each dorsal spinal root becomes a single nerve-bundle whic passes towards the periphery between two myotomes, and, reaching a lev immediately below the skin, divides into a dorsal and a ventral ramus. Fro each of these arises a cutaneous system of sparse sensory fibres. In the regio between the mouth and the atriopore the ventral ramus of each segmen slightly diminished by the loss of its cutaneous sensory component, passe downwards between skin and myotomes. At a ventral level it sends a mai branch inwards, between a myotome and the pterygial muscle; this is calle the visceral ramus. This itself divides, on reaching the parietal wall of th atrium, into an ascending and descending ramus. The ascending ramus passe up over the parietal wall to the dorsal part of the atrium just below th notochord; in the gill region it is distributed over the denticulate ligamen gill bars and anterior gut diverticulum ('liver'). Behind the pharyngeal regio the ascending rami of the corresponding segments follow the epithelium i which the gut is suspended, and ramify over the gut.

The descending visceral ramus is distributed in the tissue overlying th inner (atrial) side of the pterygial muscle. Figs. 5 and 8 illustrate the gener anatomy of the animal in the atrial region.

METHODS AND RESULTS

My conclusions are based almost entirely on material impregnated wit silver, for the demonstration of nervous elements, by the buffered silver pyridine technique. It is applied to serial paraffin sections in the manne already described (Holmes, 1947). Many valuable preparations have bee made by impregnating whole pieces of tissue, the staining procedure bein the same but with changes in the time schedule. Formaldehyde mercuri chloride was the preferred fixative but useful, and sometimes significantl different, results were obtained after the employment of Bouin's fluid and als a mixture of ethyl alcohol, mercuric chloride, and trichloracetic acid (Huber' fixative). All animals were anaesthetized in a 10 per cent. solution of pur ethyl alcohol in sea-water before fixation.

Nothing will be said in the following account of the innervation of th asymmetrical diverticulum of the atrium or of the hind gut, both of whic lie behind the atriopore. For the cells of the atrial epithelium in this regio are heavily loaded with a pigment which interferes with silver impregnation and no thoroughly reliable new observations have been made.

The neurons of the gut wall

A typical form of the nervous system of the gut wall is shown in fig. 1 which is part of a whole preparation of the gut diverticulum. In the are

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FIG. 1 (plate). A typical field showing the organization of the nervous system of th anterior gut diverticulum. Whole mount. Silver.

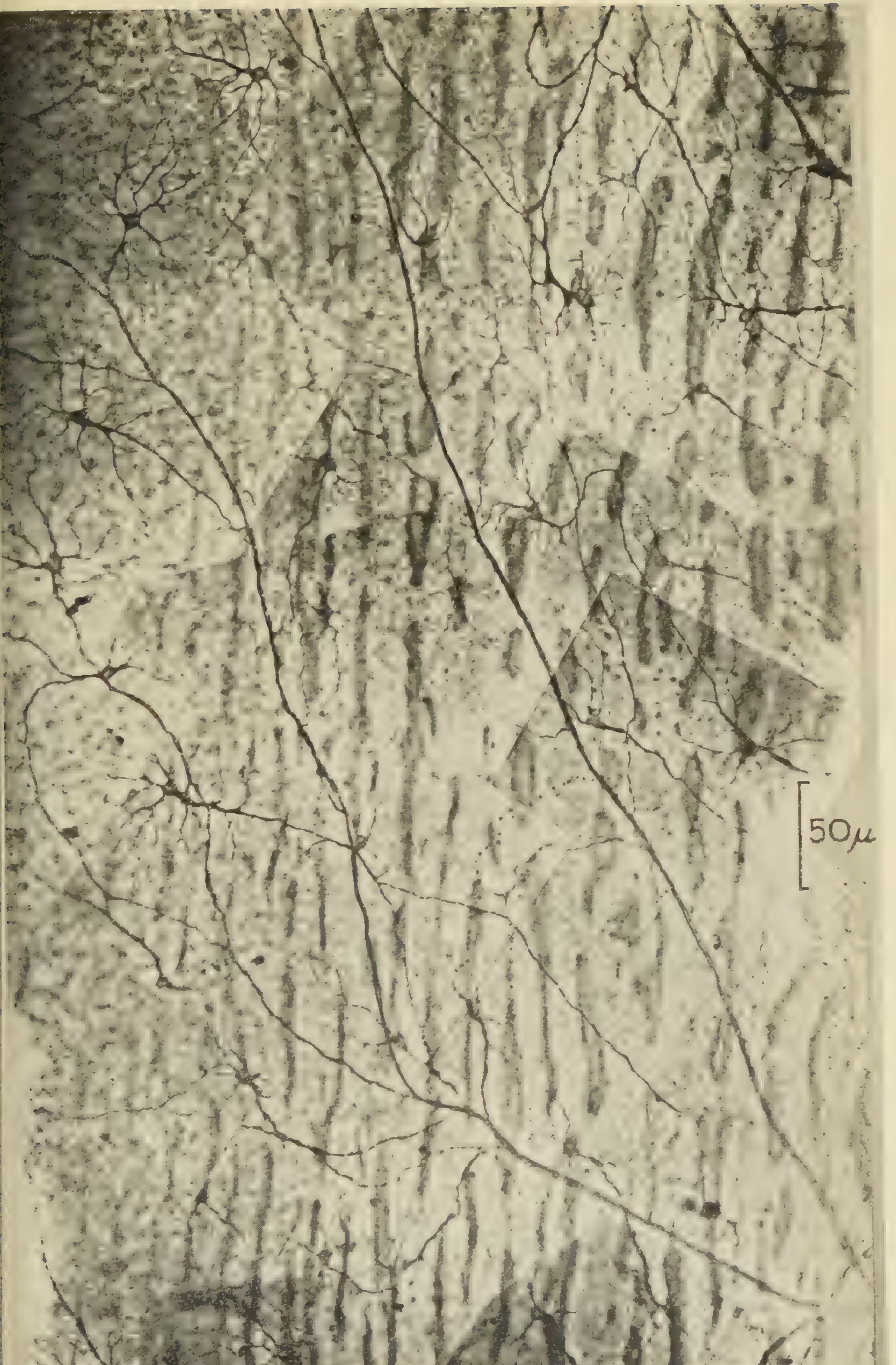

FIG. 1
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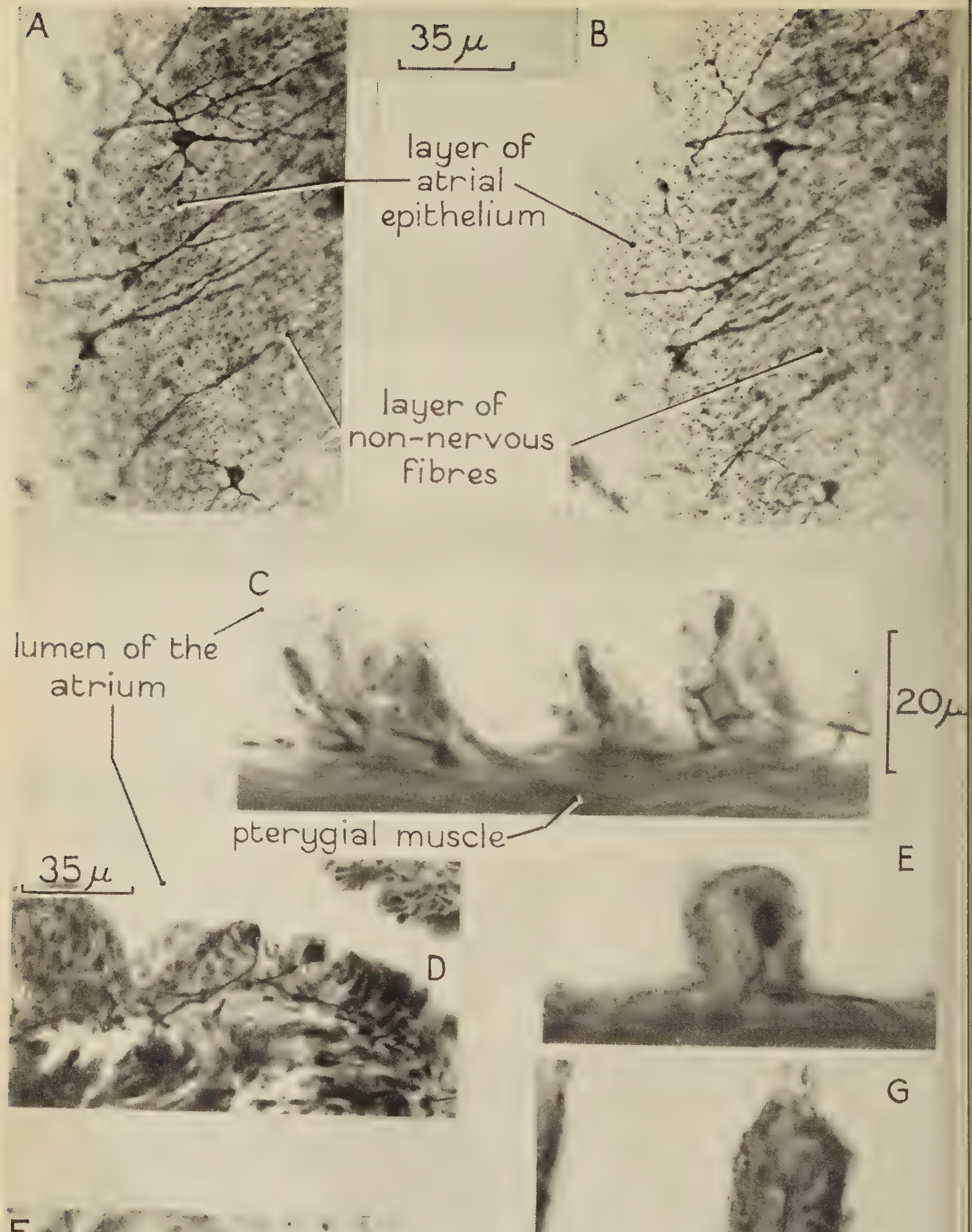

FIG. 2

W. HOLMES

illustrated lie the cell bodies of more than fifty neurons, and this is a normal concentration. Two major bundles of axons converge towards the bottom right-hand corner of the plate, and it is characteristic throughout the system that such bundles unite in the dorsal region of the atrium to the ascending visceral ramus of each segment.

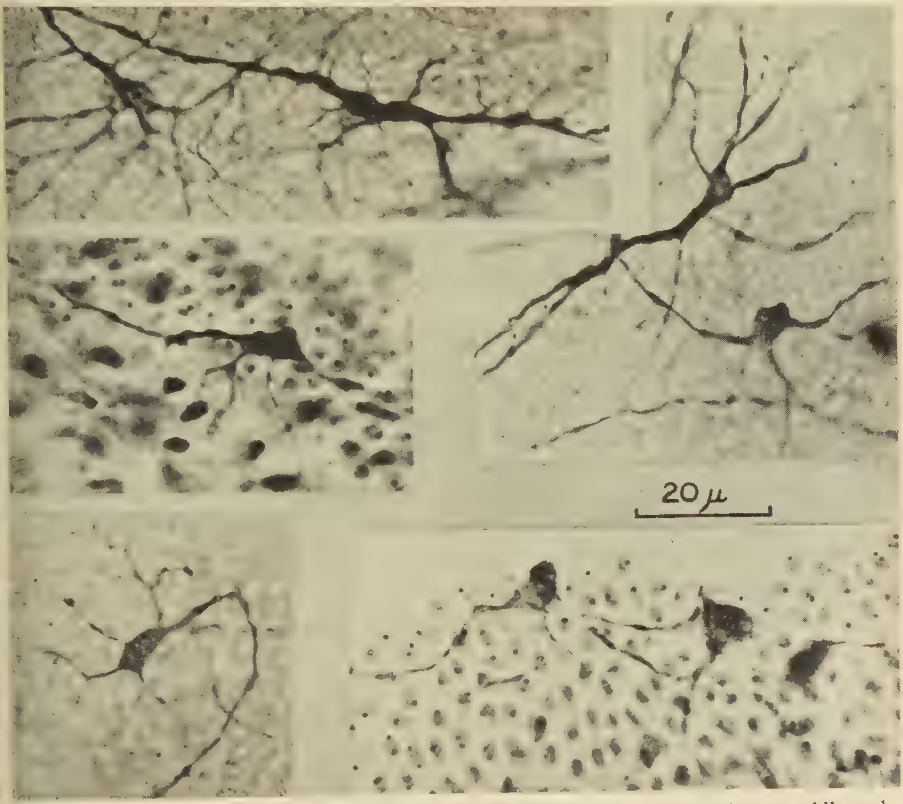

Fig. 3. Neurons in the atrial epithelium from various positions around the gut. All to the same scale. Silver.

Preparations such as this suggest that the character and relations of the visceral neurons of amphioxus are different from those found in the visceral systems of craniate animals. Individual neurons from different parts of the system are shown at higher magnification in fig. 3. The very numerous dendritic processes from the cell bodies relatively rarely enter into relationships with other nervous processes. The dendrites end in fine filaments, sometimes knobbed, which pass up or down out of the plane in which the cell body lies,

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Fig. 2 (plate). A and B. One field, at different focal planes, in a tangential section of the gut immediately behind the pharynx. Silver.

C, E, and G. Atrial papillae. Silver. All to the same scale.

D and F. Atrial epithelium and underlying muscle in the region of the atrioporal chamber. Silver. F to the same scale as C.

and it seems that these dendrites are more often terminal structures rathe than the site of synaptic connexions between their own neuron and processe from others.

There is another difference from higher animals in that the axons whic collect together to form the ascending ramus seem to be actual processes o the intrinsic neurons in the visceral system, and not the axons of preganglioni neurons.

My investigation has so far been confined to a general survey of the atria nervous system by a single neurological method. It is *suggested* above that th dendrites are terminal structures, and that many of the axons in the ascendin visceral nerves are the processes of cells in the gut wall. Both these views ar contrary to Boeke's interpretation, and further work is needed. It should b pointed out that even in mammals our understanding of the relationship between visceral neurons is not yet sufficient to provide a complete picture o the functional organization of the system. We need detailed studies of singl cells by a variety of methods.

An experimental attack on this question led to no conclusion. Animals i which several dorsal nerve roots were cut on one side of the body could b kept alive in good condition for no more than 5 days at Plymouth, and histological examination after this lapse of time showed no significant difference in cell bodies or axons on either side of the cut.

All viscera are covered, on their atrial side, by the atrial epithelium, usuall one cell thick. This arises in development as an invagination of the larva ectoderm. It is continuous also over the parietal wall of the atrium, so it ma be said to suspend the viscera, being specialized as the denticulate ligamen in the gill region. Dissection of fixed animals makes it possible to separat quite large sheets of atrial epithelium, particularly that of the denticulat ligament and gut diverticulum. Such sheets of epithelium have been impregnated with silver. The examination of these preparations, and of large number of sections, such as are illustrated and referred to later, leaves no doubt tha the cell bodies of the majority of the neurons of the atrial system lie in th atrial epithelium, and that their axons are immediately below and intimatel connected with it. Boeke discussed this suggestion, and rejected it, bu admitted that his material was poorly fixed and unsuitably prepared for cel localization (1935*b*). It may be concluded therefore that the atrial nervou system belongs to a layer which is of ectodermal origin. It would be fascinatin to study the larval development of the system.

Visceral effectors

Secretory cells form such a large part of the internal lining of the gut o amphioxus that the visceral nervous system might well have a secreto-moto component. But of this there is no evidence. Smooth muscle fibres might als be expected to be present. Histological identification of smooth muscle i particularly difficult in this animal since spindle-shaped cells are widely distributed in its primitive connective tissue (Franz, 1925). Silver impregnatio after Huber fixation picks out the larger of such cells quite clearly; around the conus arteriosus and adjacent trunks can be seen encircling fibrous bands (fig. 4, B) and Franz was, no doubt, right in describing the vessels as myoepithelial tubes (1933). The relations of the larger vessels are shown in fig. 5, and they are suspended in folds of atrial epithelium. But I have not been able to convince myself of the existence of any contacts between the atrial neurons and the fibrous elements of the vessel wall. This is not to say that such contacts do not exist.

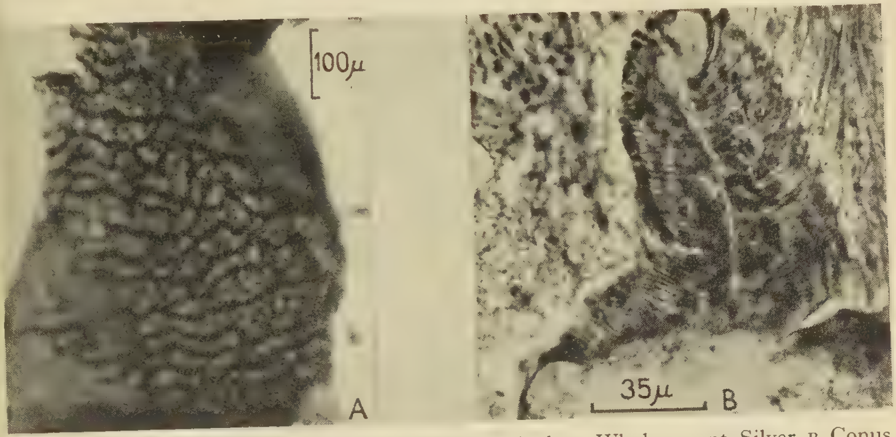

FIG. 4, A. Vascular plexus in the wall of the gut diverticulum. Whole mount. Silver. B. Conus arteriosus. L. S. at 20 μ. Silver. Fibres of (?)smooth muscle impregnated.

A network of blood-vessels forms a peculiar capillary bed in the walls of the gut (Franz, 1933). It is occasionally seen very clearly in silver preparations as in the whole mount of a portion of the gut diverticulum in fig. 4, A. Boeke satisfied himself of the presence of smooth muscle throughout the gut wall, but does not express an opinion as to whether it is concerned in vascular control or gut movement. In my preparations fibres which might be smooth muscle are found in the post-pharyngeal gut but not in the gut diverticulum. They are narrower than those surrounding the great vessels. Fig. 2, A and B are photographs of the same field in a tangential section through the wall of the gut, taken at different focal planes. Faint parallel non-nervous fibres are impregnated, and are distinct from the characteristically shaped neurons. Comparison of the photographs shows how the fibrous layer lies immediately below the nervous layer, and in the preparations one can see a suggestion that the dendritic processes of the latter pass downwards to the layer below.

Much of the literature on digestion in amphioxus omits the consideration of the possibility of muscular movement in the gut, but a paper by Reeves (1931) states decisively that such movements do take place. Having studied the Amoy amphioxus she describes and figures contractions of the gut, taking the form of peristaltic waves.

The atrio-coelomic funnels and trapezius muscle

Ray Lankester recorded all his contributions to the morphology of amphioxus in the pages of the Q.J.M.S., and amongst these was the discovery an description of the atrio-coelomic funnels (1875 and 1889). The weight o

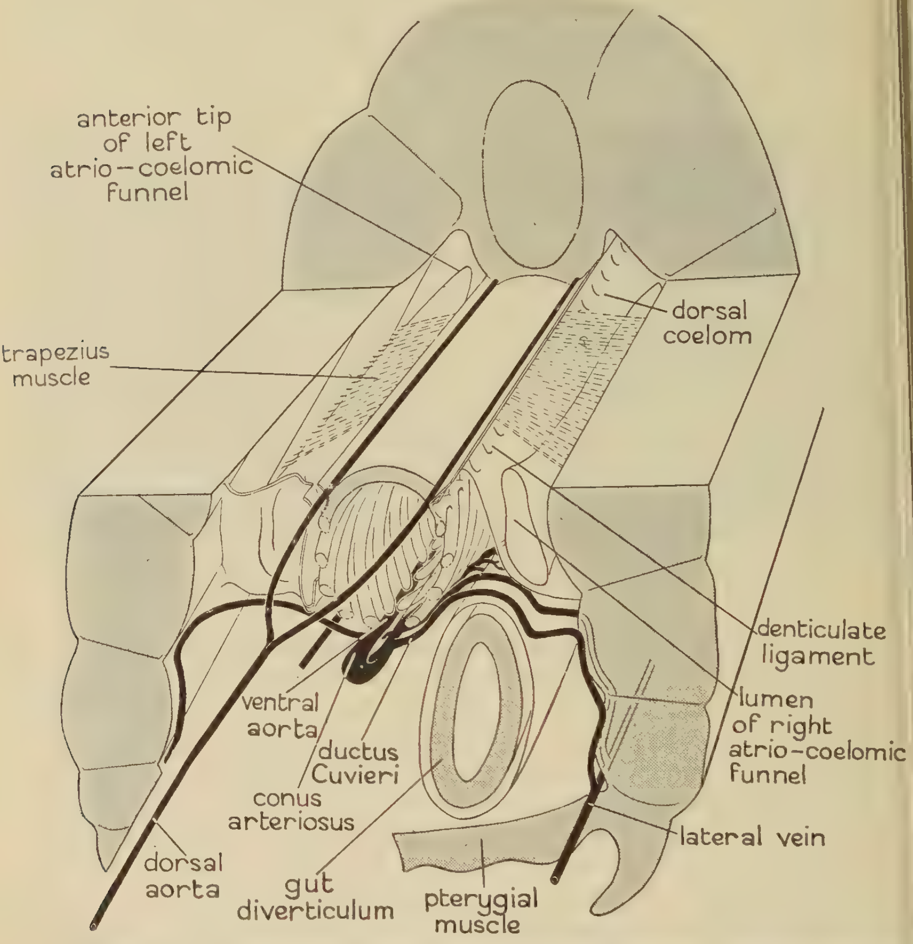

FIG. 5. Diagram of the anatomical relations of non-nervous structures in the posterior region of the pharynx and immediately behind it. The observer is looking towards the head end. The atrio-coelomic funnel of the right side has been cut further forward than that of the left.

opinion is now in favour of the view that these structures are open at their larger end only, into the atrium, and not into the dorsal coelom. But the word 'funnel' is regularly used, and it is convenient to retain it. The accompanying diagram (fig. 5) may suffice to remind readers of the relationships of the funnels. A special thin sheet of muscle overlies each funnel, as shown in the diagram, and Legros, who described its relationships, called it the trapezius muscle (1902).

I have never been able to observe the funnels in the living animal; Zarnik (1904) saw movements of the pharynx, and attributed these to contractions of this muscle; Franz (1925) suggested, as is reasonable, that the contractions result in a narrowing of the funnel as seen in cross-section. Certainly the shape of the funnel varies in different sets of fixed material. But the atrial epithelium of which the funnel is composed is always, whatever its shape, closely applied to the fibres of the trapezius muscle.

Legros (1902) and Franz (1927) have shown that the funnel of each side is innervated from an unusually large ascending visceral ramus, which passes close to the ductus Cuvieri; this ramus contributes also to the nervous system of the gut diverticulum. The fibres of the trapezius muscle are striated, though the striations, in silver preparations, are unlike those of the myotomal muscles in being much coarser and more widely spaced. Boeke, having stated the homology of the atrial system with the vertebrate autonomic, concluded that the innervation of the striated trapezius from the same source as the gut was evidence for the view that all striated muscle may have visceral motor as well as somatic motor innervation. (Boeke's paper 1935*b* is very largely a German translation of 1935*a*, but it does include some new drawings of the trapezius and its innervation.)

I wish to emphasize here the richness of innervation of Lankester's funnels, which is such that a transverse section through them seems to contain more neurons than any other comparable section in the atrial system. Figs. 6, A and B are photographs of two such sections; the cell bodies of the neurons lie in, or slightly below, the atrial epithelium; the axons lie between it and the fibres of the trapezius muscle, and very likely some innervate the muscle, though I have not seen any special end-plates. There are a number of structures in the epithelium of the funnel which might be interpreted as sensory terminations, but I am not yet satisfied on this point which, if based on purely morphological criteria, requires to be approached with scepticism. The standards accepted as valid for a morphological decision of this kind are shown in the next section.

The neurons of the atrial floor and atriopore

Several workers have described how the descending visceral ramus of each segment, in the atrial region of the body, passes as a branching transverse nerve over the pterygial (transverse) muscle which forms the atrial floor, reaching to the mid-line. There are overlaps and exchanges of axons between the branching bundles of adjacent segments (Heymans and van der Stricht, 1898; Dogiel, 1903; Franz, 1923 and 1927). The transverse nerve lies between the epithelium of the atrial floor and the fibres of the pterygial muscle, which undoubtedly receive from it their motor innervation. This was shown in my experiments on dorsal root section: the pterygial muscle on the operated side of the animal was quite different from the normal, after fixation; it was half its normal thickness, and abnormally extended along the axis of its component fibres. Evidently the motor nerves from the dorsal root not only mediate the well-known convulsive contractions of the atrial floor, but also maintain a tonic state when it is apparently at rest.

The atrial epithelium covering the pterygial muscle is modified from its simplest form in numerous patches; here the cells are elongated and project into the lumen of the atrium as papillae of various sizes; in these there is evidence of cytological differentiation within the epithelium. These cell masses are rare in the pharyngeal region, becoming progressively more numerous as one approaches the atriopore, in front of which they are most dense. They were noticed by J. Müller, and have sometimes been called the 'kidneys of Müller', or atrial glands. It is best to call them 'atrial papillae'. They were examined by a pupil of Lankester's, who found their cells capable of taking up dye particles (Weiss, 1890). Van Weel has suggested that they have a function in the nutrition of the animal, taking up by phagocytosis particles of food that have escaped from the pharynx (1937).

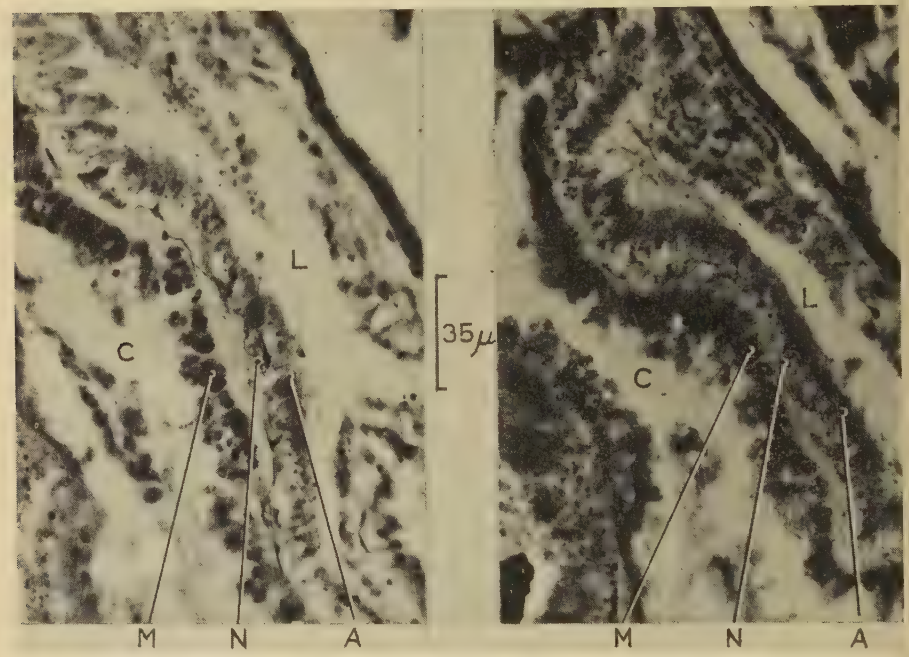

FIG. 6. Two transverse sections each containing the major part of the cross-section of an atrio-coelomic funnel. Silver. A, atrial epithelium; C, coelom; L, lumen of funnel; M, trapezius muscle; N, nervous layer.

A new study of the cytology of the atrial papillae would no doubt be fruitful; I have only gone so far as to show that they contain terminal branches from axons in the transverse nerves. Fig. 7, B is of a horizontal section through the atrial floor, close to the atriopore. A portion of the pterygial muscle comes into the bottom of the picture. The rich network of the transverse nerve-fibres can be seen, and six distinct parallel columns of cells; these are rows of atrial apillae running along the antero-posterior axis of the animal. Fig. 7, A is of transverse section through the same region, and shows that many of the erve axons pass actually up into the papillae. The innervation of the papillae shown at a higher magnification in fig. 2, C, E, and G. The two former of

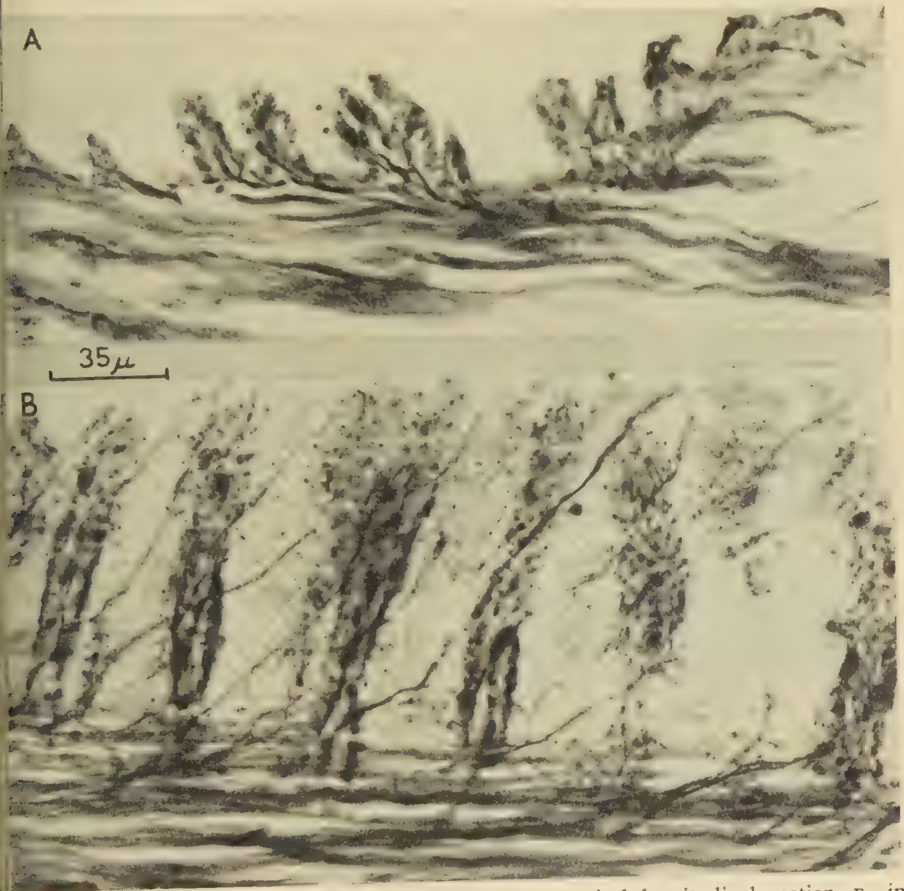

FIG. 7. Atrial papillae and their innervation. A—in vertical longitudinal section, B—in a horizontal section of the atrial floor. Silver.

these photographs illustrate what seem to be special structures connected with the nerve-fibre; they lie at the surface of the papilla or apparently, as in E, slightly within it; they are more darkly impregnated with silver than the other cells. It is reasonable to propose the hypothesis that these structures are of a sensory nature, but I have not been able to form an opinion as to whether they are sensory cells—modified epithelial elements innervated from the transverse nerve, or sensory neurons—unipolar elements whose axons contribute to the sub-epithelial plexus.

The atriopore of amphioxus is more than a mere perforation from the exterior to the atrium, for it opens first into a chamber of its own, and only through this into the atrial cavity. This was shown by Dennell (1950) sections, and he and Franz (1925) point out that the lips of the pore ar chamber are capable of adopting various shapes and degrees of closure. I fig. 8, reconstructed from serial sections, an attempt is made to show th appearance of the atrioporal region, as seen from inside the atrium, lookin backwards towards the atriopore. The fibres of the pterygial muscle, whic are parallel and transverse throughout most of the atrial floor, have a mo complex arrangement around the pore and its chamber; the muscle is muc thicker here, and muscle bands run in various planes. The complexity of th transverse nerve network in this vicinity is no doubt related to its mor complex motor functions in controlling atrioporal activity (fig. 9).

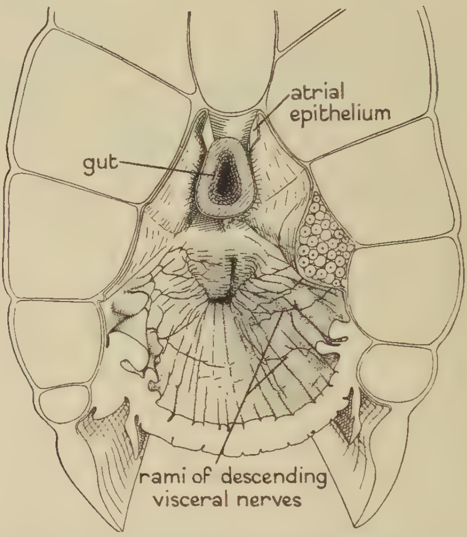

Fig. 8. Diagram of the posterior part of the atrium seen from inside. The observer is looking towards the tail end, and the gut has been transected behind the atriopore. The pore i seen as a vertical slit opening to the exterior from the atrioporal chamber, which rises abov the general level of the atrial floor.

There is evidence of the existence of a local sensory component in the atria system, with its endings in the epithelium in and close to the atriopora chamber. There are often darkly stained masses within the epithelium an at the end of axons, looking more like blobs of axoplasm than cells, and it i difficult to think of these as anything but sensory terminations (fig. 2, D) Another appearance seen round the atrioporal chamber is shown in fig. 2, F where a neuron lying in the epithelium sends out processes in several directions below the epithelial layer.

Diverse types of neuron

We have not considered here the neurons and their connexions in various parts of the atrial system such as the somatic atrial wall, the outer surfaces o he gill bars, and the epithelium underlying the ventral aorta. The neurons in hese situations are often much smaller than those on the gut wall, widely paced, and of different shapes. A good deal can be found out about them and heir connexions from the literature, particularly in the works of Heymans nd van der Stricht (1898), Dogiel (1903), and Kutchin (1913). There is no loubt that the whole atrial system is interconnected.

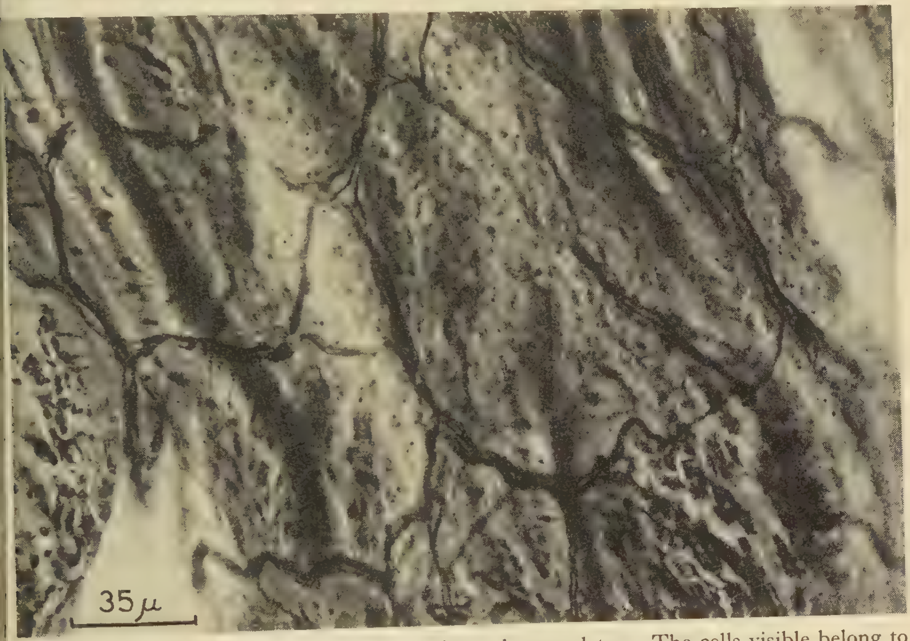

Fig. 9. Part of the nerve plexus on the atrioporal musculature. The cells visible belong to the atrial epithelium. Silver.

Concluding Remarks

In the atrium of amphioxus there is a nervous system containing, perhaps, more neurons than the corresponding region of the spinal cord. Apart from the motor division of the descending visceral ramus, which comes straight from the cord to the pterygial muscle, there is great uncertainty as to the organization of the system. If the suggestion made in this paper is confirmed, then the dorsal roots of amphioxus are partly composed of axons from atrial neurons, which are thus anatomically afferent. Boeke accepted that the dorsal roots are predominantly somatic motor, and put confidence in Ayers's claim that a visceral motor nerve comes off from the ventral roots, supplying the atrial system. Ayers's observations seem never to have been confirmed (Nicol, 1952).

Evidence has been given that there are sensory as well as motor elements in the atrial system, and it is interesting to recollect that the contents of the atrial cavity, in the living animal, are a constantly refreshed representation of its external environment. The skin of amphioxus, with its one delicate laye of epidermal cells, has only a sparse innervation, by fibres ending in un specialized terminations. We must consider the possibility that the anima takes an important part of its sensory data from the protected surface of th atrial epithelium, invaginated from outside. The atrio-coelomic funnels an atrioporal region may be special sampling organs, with a motor system enabling them to function, and a sensory equipment influencing effector either by way of the spinal cord or locally. The special activities involved in reproduction must be borne in mind, for the genital products are discharge into the atrium and out through the atriopore.

But when speculating it is salutary to remember what was written mor than a hundred years ago, by one of the first students of the nervous system of our animal:

> 'L'amphioxus . . . a été de ma part l'objet d'une étude aussi détaillée qu'il m'a ét possible, et aujourd'hui on peut en regarder l'organisation comme parfaitemen connue' (de Quatrefages, 1847, p. 145).

The purpose of this paper is to invite students of the nervous system to look again at amphioxus. Study of its organization will tell us nothing new of the animal's position in the evolutionary hierarchy. But as an early offshoot of the chordate line, retaining primitive characters, it may in its atrial nervous system have something to show of the way in which nervous units were organized at this early stage, making the success of the craniates possible. And it will be profitable to compare it with lower forms, coelenterates, echinoderms and hemichordates, rather than looking forward only, to a complex end-product as seen in the mammalian autonomic.

Part of this investigation was carried out in the Laboratory of the Marine Biological Association at Plymouth, and I am most grateful to Mr. F. S. Russell, Dr. H. G. Vevers, and all other members of the staff who gave me help so generously.

I wish to thank Mr. Andrew Packard for the painstaking care with which he made the line drawings of the reconstructions which appear as figs. 5 and 8.

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Physical Factors involved in the Vertical Migration of Plankton

By JOHN E. HARRIS

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SUMMARY

It is suggested that vertical migration is an inescapable consequence of the use of light by planktonic forms as a means of attaining a level within the euphotic zone; no other sense organ appears capable of producing this result. The mechanism of light response in *Daphnia* fulfils the necessary requirements for vertical migration, and field observations on other species suggest the presence of similar if not identical physiological features in them. These features are a positive photokinesis which is combined with negative geotaxis at low light intensities; at a critical higher value of light intensity a typical dorsal light reflex cuts off the vertical rise. The dorsal light reflex is probably a response to illumination of the eye within the critical cone of submarine illumination, and is therefore not related to the direction of a particular light source such as the sun.

THE causes underlying the daily rhythm of vertical migration have been a subject for active speculation for nearly 80 years now. In one of the latest contributions to our knowledge of this fascinating problem Hardy (1953) makes the interesting suggestion that it was evolved as a means of providing for the horizontal distribution of organisms which have very limited powers of active locomotion, since the intensity and direction of horizontal currents in a large body of water will vary considerably with depth.

The selective value of such a mechanism is clearly very considerable, but the implication that it was evolved many times over primarily for this purpose is perhaps open to question. As a result of a fairly extensive but unfinished laboratory investigation into the physiological basis of this migration, the results of which will be published elsewhere, a number of general considerations have been raised which it may be profitable to present here, since without invalidating Hardy's theory, they suggest that vertical migration may have originated for somewhat different reasons.

It has been taken for granted by most workers on the subject, and a very large number of field and laboratory studies support the view, that light is the main factor in producing this vertical migration. The theory of Michael (1911), and the careful and detailed analyses of such workers as Russell (1925, 1926, 1928), Ullyott (1939), and Clarke (1933) suggest that the organisms move into, or remain within, a zone of a definite intensity of illumination, and that with a change in intensity of overhead light, the organisms follow to a greater or lesser extent the corresponding vertical shift in this zone.

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Even earlier than the exact field-studies mentioned, Ewald (1910, 1912) ha investigated the vertical movements of *Daphnia* in the laboratory and ha suggested a physiological mechanism for producing this result; the later wo of Rose (1925) and Clarke (1930, 1932) provided further evidence for i probability. As our own experiments with *Daphnia magna* have pointed this same conclusion, it seems worth considering its implications for th organism quite apart from its relationship to vertical migration.

It seems reasonable to start from the assumption that a planktonic organism, being by definition one with very limited powers of locomotion, canno seek out its food over considerable distances. Like the sessile filter feede it must rely rather on 'encountering' it. For the smaller members of th animal plankton, encountering a sufficiently dense food supply to mainta them must inevitably mean spending a certain minimum proportion of the time in the euphotic zone where small phytoplankton can grow and reproduc Even the larger planktonic animals which may feed on other members of th animal plankton will have a better chance of encountering their food if the also are adapted to visit or occupy the same level.

This euphotic zone extends over a considerable depth depending on th transparency of the water. Its upper boundary reaches the surface, thoug there is some evidence that the optimum light intensity for photosynthesi is appreciably below the value at the surface under conditions of intense sola illumination. The depth of the compensation point where photosynthesis jus balances respiration represents the lower boundary of the euphotic zone an is given by Jenkin (1937) as 45 metres in the English Channel, by Clark and Ostler (1934) as 40–50 metres in the Gulf of Maine; the light intensit at this compensation point is quoted by Sverdrup, Johnson, and Fleming (1942) as being in the region of 0·1 gm. cal./cm.^{2}/hr., equivalent to abou 600 lux. This is about 0·005 of the light intensity at the surface on a brigh day and suggests that in the clear waters of the open ocean the compensatio point may be as far down as 150 metres.

The figures above are given as rough approximations only; their values vary considerably with time of day and season of year, with latitude and weather conditions. The luminous equivalent of light in terms of energy varies with the wave-length and consequently with depth of penetration and 'colour' of the water—and the availability of this energy may depend to some extent on the photosynthetic pigment of the plant cell. It appears, however that the main source of food in open water is to be found in the zone extending from the surface down to 50 or 100 metres in depth. Those animals which make use of this food source either directly as herbivores, or indirectly by feeding on the herbivores, will need to spend much of their time feeding in this zone.

There are obviously many ways of achieving this end. The simplest of all would be provided by a floating animal which because of its low specific gravity would necessarily remain indefinitely in the surface waters. But there are few such forms—the Siphonophora are perhaps the most obvious, though many of them do not float at the surface—and the disadvantages of such a specialization are manifest. Continuous and violent mechanical disturbance would probably make filter feeding very ineffective. It is also commonly found that the population density of phytoplankton falls off appreciably in the surface layers; Zobell and Anderson (1936) found the greatest number of diatoms off the California coast at a depth of 30–40 metres. Most important would surely be the intense competition in these surface layers for the limited plant food supply. The work of Harvey, Cooper, Lebour, and Russell (1935) and the later calculations of Fleming (1939) have shown how delicate is the balance between grazing animals and their food supply; if to this situation we add the problem posed by Hardy (1953) of the impossibility of horizontal redistribution of the grazing population except by the differential effect of currents at different depths, we can see that animals found in the surface will either be temporary visitors or, if permanent, will be carnivorous predators on the migrating animal population—as with the siphonophores previously quoted.

What is needed, therefore, is a mechanism which maintains the animal near the surface but not in it. When, however, we consider how this is to be achieved we find that there are surprisingly few effective ways of doing so. It is essential that the organism should remain within access of this feeding zone; random movements or even continued downward movements for a long period would inevitably lead to starvation and death. Maintenance at an approximately fixed level—even if only for part of the 24 hours—can be achieved in two different ways. The organism may locate itself by some fixed plane of reference, which in this case must necessarily be the surface or the substratum. The lateral line organs of fishes could and probably do serve this purpose near the sea floor, but Pumphrey (1950) has pointed out their relatively short range except in large animals; similar objections would apply to the use of electric organs, and neither would be of value in locating the animal with reference to a disturbed water surface. Optical methods depending on appreciation of distance would be equally ineffective for microscopic animals, even though the human diver can easily estimate his approximate depth below the surface by such means.

There remains therefore a second type of mechanism depending upon some property of the environment which varies with depth. Pressure is the obvious one to choose, as its relationship to depth in water is consistent and exact, and animal sense organs which respond to pressure changes are well known.

Such sense organs, however, are present only in large and relatively complex animals. Best known are the air bladders of teleost fish (Harden-Jones and Marshall, 1953) and the bubble receptor of the hemipteran *Nepa*. The compressibility of biological tissues is essentially the same as that of water, so that only a gas space of some type could function as a suitable pressure receptor. But the equilibrium of any such bubble in contact with actively respiring tissue is unstable (as is well known in the use of the air bubble as a physical gill in insects). Either it must be renewed from time to time by contact with air—as presumably happens in *Nepa*—or relatively elabora secretory mechanisms must maintain its gas content qualitatively and quant tatively unchanged. Though such secretory mechanisms exist in the g glands and *retia mirabilia* of fishes, they are unlikely to occur in small planktonic forms, and even in fishes the equilibrium they provide is a short ter rather than a long term one. It is not surprising that Hardy and Bainbridg (1951) failed to demonstrate a sensitivity to pressure in typical marine planktonic forms, though they have possible evidence for its existence in som specialized planktonic larvae.

The chemical characteristics of an aquatic environment show no larg systematic variation with depth, and sense organs responding to salinit oxygen, &c., would be ineffective for depth control. The same is true temperature variations, though there is a good deal of evidence that temper ture may play an important secondary role in the control. There is in fa only one remaining property which varies consistently with depth and, i addition, is itself a principal determining factor in the abundance of pla food; this of course is variation in light intensity.

Sensitivity to light is a property which seems to be present in all organisn to some extent—even in those possessing no obvious light-sensitive pigmen Viaud (1951) has presented evidence for the presence of two distinct ligh sensitive mechanisms in animals as widely separated phylogenetically as th rotifers, planarians, and *Daphnia*; he believes that this phenomenon is eve more widespread. One of these photosensitive mechanisms shows its max mum activity in light of short wave-length and produces a photokinetic effe (in the sense of Fraenkel and Gunn, 1940) on some characteristic activity the organism—usually demonstrated on rate of locomotion. The other show a maximum activity at wave-lengths in the yellow–green region of th spectrum and in the three species studied by Viaud produces a differenti effect, for example on steering mechanisms or phototactic responses.

It can scarcely be a coincidence that in a large proportion of anima possessing eyes, and even in those where eyes as such are not present, bu photosensitivity of this second, specialized type exists, the maximum effectiv ness of incident light, and correspondingly the maximum absorption of th visual pigment, lies in this yellow–green region—precisely the region the spectrum which shows the greatest penetration in water. It suggests ver strongly, as Walls (1942) has pointed out and many earlier investigators hav assumed—Bayliss, Lythgoe, and Tansley (1936) for vertebrates—that th visual purples and similar pigments were evolved primarily for use in wate A photosensitive mechanism with its maximum effectiveness in this regio would be an extremely useful basis for control of depth in an aquatic form

The relationship of the photochemical effect to the light intensity perhap provides another clue to its usefulness as a depth gauge. The effect has bee shown for several organisms to be approximately proportional to the logarith of the light intensity—a common property among sense organs of other typ which approximately obey the Weber–Fechner law. Light penetration in olumn of water is exponentially related to the depth of the column, consequently a sense organ responding proportionally to the logarithm of the light intensity will give a linear response with depth. The sensitivity and accuracy of control of vertical movement by this means will thus be reasonably constant over wide limits.

All these considerations indicate the value of photosensitive mechanisms as a means of maintaining the planktonic organism at a suitable depth for feeding—and the earlier arguments suggest that there is no other obvious practicable way of achieving the same result. If so it may well be that vertical migration was in the first place an inescapable concomitant of the use of photosensitive organs for this purpose.

Organisms did not evolve vertical migration habits in order to survive; rather, they acquired them as an accidental consequence of using light as a means of depth control.

THE MECHANISM OF CONTROL BY LIGHT

A mechanism of the sort we have described, in which the intensity of stimulation of some system governs the level—an equilibrium point—which the organism occupies, is in modern jargon a homeostatic mechanism and the control is exercised by a negative feed-back of the sense organ on an activity which in the absence of such feed-back tends to displace the equilibrium continually in one direction. In *Daphnia*, and presumably in other planktonic forms, this continual displacement is in fact a vertical rise; the controlling mechanism cuts off this vertical rise when the appropriate level of light intensity is reached. To be effective, there must be an active or passive tendency in the opposite direction (i.e. to fall) when the control is working. This results in an oscillation about the equilibrium point which in a successfully balanced system is a stable oscillation. In terms of the familiar governor on a steam-engine, the steam pressure, uncontrolled, would bring about a continually increasing speed of rotation. But the governor at a certain speed closes the steam-valve, and when this happens, friction and the working load on the engine slow down the rate until the now more slowly rotating governor allows the steam-valve to open again. The system oscillates about its equilibrium value to a greater or lesser extent depending on (1) the lesser or greater sensitivity of the control, (2) its all-or-none or gradual character, (3) secondary damping mechanisms which slow down the rate of change of speed—the flywheel of the steam-engine.

In a planktonic animal there is no *a priori* reason why the continually active locomotor movement should not be a downward one, cut off by a sense organ at a certain minimum light intensity. The reverse movement—rising—would then be passive, and the animal would be a floating organism which continually swam down to the equilibrium depth. The difference between this second method and the first is that the feed-back of the sense organ must now work in the opposite way; stimulation of the eye must inhibit a centre which itself tends to inhibit the downward locomotion.

When light intensity diminishes, the inhibitory action of the photosensitiv centre disappears, and the inhibitory action of the secondary centre is the allowed to reduce the locomotion. Stated in this form, the system is clearl one stage more complex than the first mechanism, where increase of ligh intensity stimulates the photosensitive centre to inhibit the locomotor centr

There is, however, a third way, simpler than the above, of achieving th desired result in a 'floating' animal. If locomotion is directly excited by ligh intensity (and is in a downward direction) it will gradually decrease as th animal descends until it is just equal to the tendency to float up; the anima will asymptotically approach its equilibrium point.

We may perhaps eliminate the second method partly on the grounds o its unnecessary complexity. But there must be some cogent reason for th use of the first method rather than the third, especially since simple positiv photokinesis is a very widespread property in animals—and particularly i planktonic forms.

One obvious objection to an active downward movement stimulated b light as the only mechanism of depth control is that the organism would the spend the hours of darkness entirely in the surface. Equally significant, however, is the fact that, if the swimming speed downwards is governed by ligh intensity, the equilibrium position would then vary with the specific gravit of the animal, which might change considerably with nutrition and wit phases in the reproductive cycle. Thus if the rate of passive rise with a specifi gravity of 0·95 were 1 cm./sec., and the equilibrium depth were to be 1 metres below the surface, then at this light intensity the swimming rate woul be 1 cm./sec. downwards. Feeding might change the specific gravity to 0·98 when the rate of rise would be only 0·4 cm./sec. and equilibrium (if swimmin speed is linearly related to light intensity) would now be attained at abou 23 metres, in ocean water of extinction coefficient 0·08 and with the surfac illumination unchanged.

Daphnia magna does in fact use active downward swimming to take i out of the lighted surface waters if a trapped air bubble makes it lighter tha water. But this is an emergency mechanism developed from the much mor effective depth control actually employed. Most planktonic organisms ar heavier than their aquatic environment; their normal rate of swimming i the dark is just sufficient to oppose sinking, and distribution is more or les random in the water column throughout and perhaps for some distance below the euphotic zone. In light, more rapid (upward) swimming occurs until critical light intensity is reached, when an active cut-off mechanism intervenes which will of course be independent of the density of the animal.

This reaction at a critical light intensity may take one of two forms. Th simplest is a direct inhibition of the upward movement; the animal sink passively until a further burst of swimming starts, the orientation remaining vertical throughout. There is some reason to believe that this inhibition o upward swimming is more primitive and probably much more widespread than the second reaction which, in animals which show both types, appear o occur at the same critical value of the light intensity. This second response n *Daphnia* takes the form of a relatively sudden reorientation of the animal so that it swims horizontally under the influence of a typical 'dorsal light reflex'.

Daphnia provides one of the clearest and most easily demonstrated examples known of the dorsal light reflex. In a tank illuminated by a point source of light of moderately high intensity the animal will orient itself so that its dorsal surface faces the direction of the incident light. This phenomenon has been recorded for a large number of different swimming animals, and in the more primitive groups of the Crustacea which do not appear to possess statocysts it is by itself capable of orienting the animal in what we may consider its normal plane.

Daphnia can readily be made to swim horizontally (which I believe to be their normal orientation in the field) in a jar illuminated either by an overhead point source or by a beam of parallel rays—this being equivalent to an infinitely distant point source. One might assume from this behaviour that one particular vertically directed ommatidium or group of ommatidia is functioning to produce a simple telotaxis—the light-compass reflex. But the explanation is not quite so simple, since *Daphnia* will maintain an accurately horizontal path when the whole of a large water surface is uniformly lit by a ground-glass plate just above the water surface, or when an open tank is placed out in diffuse daylight. Let us consider the optical features of such a situation.

Any organism under water receives light through the water surface only within an inverted cone of rays whose semi-vertical angle is the critical angle (= 49°), and whose axis is perpendicular to the water surface. Rays falling on the eye outside this cone are derived from internal reflections at the surface, and will presumably be of much lower intensity. An animal with high visual acuity and a wide visual angle will consequently 'see' the surface as a brilliantly lit circle immediately overhead, and always subtending an angle of 98°. This is a unique type of visual image which, whatever the movement of the animal, never changes its size, but only its position on the receptor surface. It might well be expected to elicit a highly specific response, as compared with other visual images (fig. 1).

If the axis of the eye is fixed relative to the body, rotation of the animal as a whole will change the position of this circular image on the retina so that the animal can take up any definite orientation to the vertical. This would be particularly true of an animal with a single median eye, but it is also true that a fish with a dorsal field of vision, by adjusting its position so that the two visual fields are symmetrically illuminated, could readily orient itself with the axis of the body accurately horizontal. Such a method would be particularly useful to fishes living in relatively shallow water; the dorsal light reflex has been reported in such fishes from marine and fresh waters by Breder and Harris (1935) in *Monacanthus hispidus* and by von Holst (1935 and 1950) in *Crenilabrus*, *Pterophyllum*, &c. It is important to note that this method of orientation by light passing through an interface from a mediu of low refractive index to one of high refractive index, is based on illumin tion from the sky as a whole and not on direct sunlight. In the sea, whe the water surface is rarely undisturbed, such direct rays from the sun a almost completely broken up by the irregular refractile boundary; the critic circle of illumination from the sky, though its boundary would be less sharp defined than in a smooth pond, would still be sufficiently recognizable serve as a means of orientation.

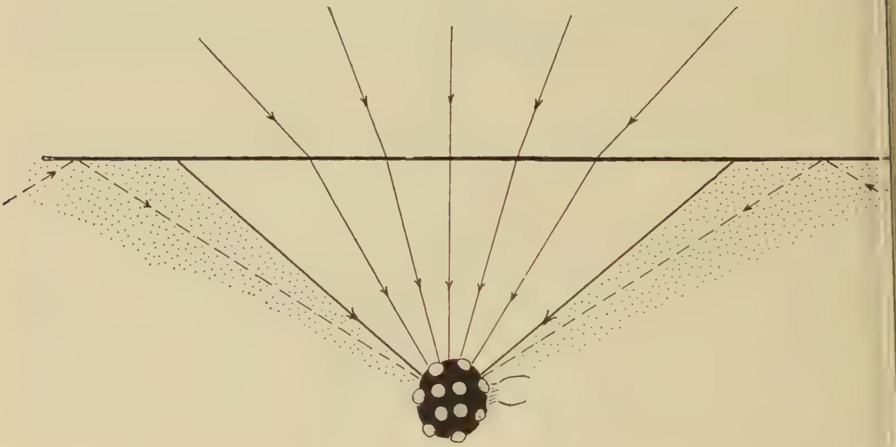

FIG. 1. The critical cone of illumination in relation to the eye of *Daphnia pulex*. Rays enterin the eye at an angle greater than 49° to the vertical are indicated by dashes; the stipple ommatidia are poorly illuminated, since such rays are the ones that are internally reflecte at the water surface.

In *Daphnia*, however, and in most planktonic Crustacea, the visual acuit is very poor. According to Heberdey and Kupka (1942), *D. pulex* possesse only twenty-two ommatidia spaced over the surface of a single median ey of roughly spherical form; the angular separation between adjacent ommatidi is about 55 degrees. The critical cone of surface illumination will consequentl illuminate a maximum of four ommatidia symmetrically, or a minimum o one if this is pointing vertically. The boundary of the critical cone—the circumference of the illuminated circle—will only be accurately delineated an centred on the visual system if this system possesses a scanning mechanism for oscillating the eye about its 'correct' equilibrium position. Such a scanning process is very characteristic of *Daphnia*; Hess (1913) described the eye as undergoing slight twitches about its mean position. Munro Fox (1949) commented on similar oscillatory movements of the eye in *Triops* and suggested their scanning function, though not in connexion with the orientation mechanism.

Scanning of the image is in fact a widespread and well-known property of eyes of low visual acuity. An extreme case is provided by Exner's (1891) famous example of the eyes of *Copilia* which have only one visual element; this oscillates rapidly in the focal plane of the cuticular lens. Though an indifferent organ of vision, such a mechanism could quite adequately serve to produce orientation with respect to the vertical. The characteristic phototelotaxis of many insects is obviously related to this method of use of the visual elements; the alternative coupling of a 'compass pattern' in the central nervous system with either a light source or a gravitational receptor is clearly the mechanism underlying the orientation dance of the honey bee (von Frisch, 1946).

Rotation of the body into the correct orientation in relation to the light is accomplished in *Daphnia* entirely by changes in the movement of the antennae.

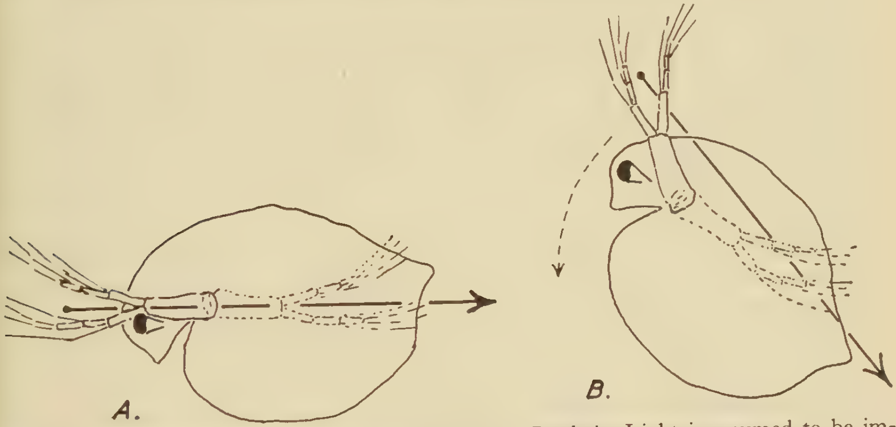

FIG. 2. The mechanism of the dorsal light reflex in *Daphnia*. Light is assumed to be immediately overhead; the eye rotates so that its dorsal surface is illuminated. In sketch *A* this involves equal tension of the dorsal and ventral eye muscles; the antennae consequently beat along the line shown by the heavy arrow, which represents the force exerted on the water. In sketch *B* the ventral eye muscle is contracted; the antennae respond to this by a dorsal postural displacement, and the resultant force on the water will rotate the animal in the direction shown by the dotted arrow.

The normal beat of these limbs is somewhat like the movement of the arms in the 'breast stroke' in swimming; from their extreme forward position at the beginning of the power stroke, the antennae are retracted outwards and backwards in the horizontal plane. (The light is assumed to be vertically above the animal, which is swimming horizontally with its dorsal side uppermost.) The resistance to forward movement of the animal is almost entirely horizontal viscous drag which can be assumed to act through a centre of pressure—not necessarily identical with the centre of gravity. In the 'neutral' position, when the orientation of the animal is not being changed, the propulsive force of the antennal beat is also horizontal and passes through this centre of pressure; the result is a simple translatory motion (fig. 2).

Pitching movements, causing rotation about a transverse axis tilting the head upwards or downwards, are produced by an alteration in the posture of the antennae. The basal joints are abducted (or adducted) by a tonic contraction superimposed upon the normal beat, and the limbs thus carry out their effective and recovery strokes inclined to the ventral (or dorsal) side.

The propulsive force now acts below (or above) the centre of pressure, tiltin the body 'head upwards' (or downwards).

If a large specimen of *D. magna* is transfixed through the brood-pouch b a long pin it can be held suspended in water lying on its side with the limb quite free to beat. On the stage of a binocular microscope such a preparatio can be watched while it is illuminated by a concentrated horizontal beam from a microscope lamp. If the light is incident on the dorsal surface of th animal the limb beats can be seen to be carried out in the neutral position and whenever the antennae are at rest for an appreciable time they will poin directly forwards.

If now the light source is moved around so that it strikes the animal mor at the anterior end, the limb posture changes towards its dorsal position eve when the limb is extended at rest. The currents generated when the limb i in motion, while still parallel to the longitudinal axis of the animal, can readil be seen to be shifted dorsally, tending to rotate the animal about the pi until the light is incident on the dorsal surface once again. This is clearl the mechanical basis of the dorsal light reflex.

This tonic postural response of the antennae is, however, not a direc response to the direction of illumination. Radl (1901) and later Hess (1913 and von Frisch and Kupelwieser (1913) have described reflex motor responses of the eye to light. As is well known, the eye is held in position by fou muscles, which can rotate it either about a transverse axis or about a dorso-ventral one through an angle of nearly 180 degrees. By this means the eye itself produces a 'telotactic' response, always attempting to attain a position such that the light falls upon its (normally) dorsal surface. Unless this is also the dorsal surface of the body, this means that the eye muscles will shorten asymmetrically; the limb posture is mediated by the tension on these eye muscles. This is clearly shown in the transfixed animal described above, since eye movements and limb positions can be studied simultaneously. If the eye is made to rotate through a very large angle, its muscles appear to fatigue at intervals. Whenever this happens the temporary relaxation of the eye rotation is always accompanied by a change in the tonic posture of the limbs, even though the incidence of light on the animal is unchanged.

The sequence: direction of light → rotation of eye → posture of limbs, resembles in a striking fashion the orientation responses of the higher vertebrates. In them, the primary sense organs for orientation are the labyrinths; one of their characteristic actions is to produce a rotation of the head. Limb posture is secondarily changed by the consequent stimulation of the proprioceptors of the neck muscles. This indirect control of limb posture is not difficult to understand in tetrapods where head movements on the neck are much freer from mechanical restraint than are movements of a large and heavy body, but it is less easy to see the reason for a similar indirect control in *Daphnia*.

One possible explanation may be suggested; in order to clarify it we must consider another characteristic feature of the response of *Daphnia* to light.

In low light intensities, and in red or green light, all of which fail to elicit eye rotation, *Daphnia* swims vertically upwards at a speed which varies directly with the light intensity. This common characteristic of planktonic forms undoubtedly helps to prevent the animal from being lost by downward movement during the hours of darkness and is also responsible for the dawn rise.

It is at first sight plausible to ascribe this vertical upward swimming to the fact that the resting posture of many planktonic forms is with the swimming limbs in the extended posture, i.e. at the beginning of the effective stroke. The animal, being heavier than water, sinks against a viscous resistance which is largely provided by the fine setae of these swimming appendages; these are situated so far forward that the body sinks tail first. Consequently any swimming stroke from this position will necessarily propel it upwards. However, once swimming becomes fairly continuous this resting orientation is no longer automatically maintained; if the animal is to continue to swim vertically upwards, a postural response of the limbs to variations from the vertical orientation must be provided.

This particular postural response is not a response to light—since we have found that maintained vertical swimming can occur when a dim light is placed underneath the tank as well as when this (or a bright green light) is overhead. It can only be a proprioceptor mechanism of some sort, dependent either on the tension in certain limb or body muscles under gravity or on the action of some statocyst. Bidder (1929), in an interesting note, suggested that the limb muscles themselves functioned in this manner but, while this could obviously be true while the animal was sinking at rest, it would not work when the animal was swimming rapidly; gravitational drag would then be very small compared with the viscous drag of the water on the body surface, and the animal would move in any direction.

Since no statocyst or similar organ has ever been found in *Daphnia*, some structure must function as a gravity receptor to maintain the limb posture which produces vertical swimming. It is not unreasonable to suggest that this structure is once again the eye. We know that differential tension on the eye muscles produces a change in limb posture even when it is not related to the direction of the light source, as when the phototactically oriented eye relaxes at intervals; if the very mobile eye differs even slightly in specific gravity from the blood which bathes it, it could readily function as a statocyst by its normal mode of action on the eye muscles, so long as it did not produce active compensatory rotation to light. This is true at low white light intensities, and even at high intensities in red and green light.

The use of such a mechanism for orientation to gravity may explain the indirect method of orienting the animal to light. It would theoretically be quite possible to produce an independent mechanism for the dorsal light reflex, using a fixed eye for the purpose. But in practice the combination of the two mechanisms into one provides an excellent basis for the observed behaviour of *Daphnia* in laboratory experiments on vertical migration. At low light intensities the orientation is geotactic and the animal swims u towards the surface; as this 'dawn rise' brings it into a higher light intensit the phototelotactic eye responses come into play and the animal now swim horizontally under the action of a dorsal light reflex.

In their very careful and detailed study of the responses of *Daphnia* to ligh von Frisch and Kupelwieser (1913) describe another interesting response o the eye. Its orientation is primarily determined by the direction of light fallin on it, but there is a secondary effect of intensity. In experiments where th light was incident on the dorsal surface of the animal, the authors say tha a diminution of the light intensity causes a slackening of the ventral ey muscle.

Reference to fig. 2 will show that this will produce a ventral postura response of the antennae: the head will be tilted upwards and the anima will swim towards the surface, not directly but in a path inclined to th horizontal at an angle determined by the change of light intensity. Thi modification of the dorsal light reflex will therefore itself tend to maintai the animal in a zone of constant light intensity and will provide for activ downward as well as upward movements when this zone of constant intensit alters in depth. The simple mechanism involving inhibition of upward swimming will only allow active motion upwards; an increase in light intensity i such circumstances would only result in slow passive sinking.

In *Daphnia*, as we have seen, the factors concerned in vertical migratio are relatively complex. A positive photo-orthokinesis is responsible for movement upwards in low light intensity, since it is combined with a geotactic orientation. This upward swimming brings the animal into a zone of highe light intensity, and at sunrise the rapid increase in light intensity will accentuate this. At some critical level orientation of the animal under a typica dorsal light reflex occurs, and the effect of change of light intensity on the orientation serves to maintain its position in this critical level of constant light intensity, moving upwards or downwards with the movements of this zone.

To what extent other planktonic organisms resemble *Daphnia* in these physiological mechanisms remains to be seen. Eye orientation is present in *Diaptomus*, according to Hess (1913), but is obviously not a factor in many organisms showing diurnal migration. Considerable differences between species and even between members of the same species at different stages in their life-history may still be explicable when we recall that the adaptation time to changes in light intensity has not been considered in this account. With photokinesis and oriented swimming working sometimes in the same direction (upwards) and sometimes in opposite directions, there are many possible variations in the pattern of behaviour.

Daphnia magna, typically a shallow-pond form, has not been known to show—or perhaps to have any particular use for—vertical migration in the field. The fact that its behaviour provides the necessary elements for such a migration is in itself perhaps a strong argument for the hypothesis presented in this article—that vertical migration is a secondary consequence rather than a primary feature of planktonic life. In fact, *Daphnia magna* has been shown to exhibit an almost perfect vertical migration cycle in the laboratory (fig. 3) under conditions which simulate those in the field, and which incidentally eliminate any participation of a diurnal physiological rhythm. Also, von Frisch and Kupelwieser (1913) pointed out that the orientation response of the eye did not occur in red and green light, and was most marked in blue, which is in excellent agreement with Ullyott's (1939) field observations that the depth of *Cyclops strenuus* is better correlated with the penetration of blue light of 4,000–5,000 Å. wave-length than with any other colour. The inconsistency of these results with those of Viaud (1951) remains to be resolved.

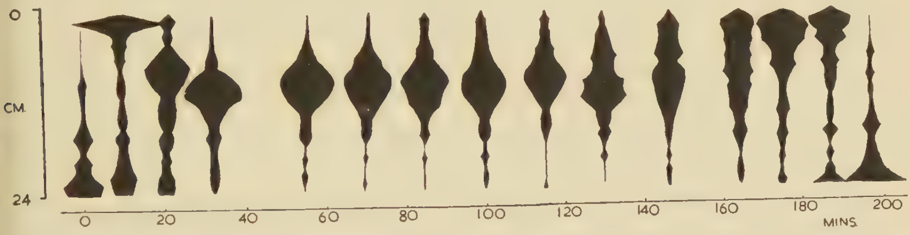

FIG. 3. An abbreviated record of a laboratory experiment on the vertical movement of a population of *Daphnia magna*. An overhead light source was varied in intensity through a typical diurnal cycle, compressed into a period of 5 hours, only 3 of which are recorded on the diagram. The *Daphnia* were swimming in an indian ink suspension which was equivalent in light absorption to a column of about 20 metres of lake water.

The present argument adds an interesting point to a paradox put forward by Pumphrey (1950), who maintains that statocysts and other gravity receptors were primarily organs of vibration sense and that their topographical and analytical function was secondary. So far as the eye is concerned, it appears that its primary function is to serve as an organ of orientation—even if in some rather crude way as in *Euglena* and the blowfly larva. In *Daphnia* the mechanism is basically the same, though more complex in detail. In so far as the effect produced on a photosensitive structure is a function of light intensity, such a mechanism is well suited to bring an animal into, and to maintain it in, a zone of optimum light intensity. If statocysts began their career as vibration receptors, it was because at that time eyes served as organs of orientation.

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Undulatory Propulsion

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SUMMARY

1. Typical undulatory progression over a rigid environment depends on three fundamental factors:

(i) Internal bending couples change the lateral curvature of each region of the body to that previously characteristic of the region lying immediately anterior to itself.
(ii) The phase of lateral bending varies along the length of the animal's body.
(iii) The presence of external restraints prevents all regions of the body from moving along any path other than one tangential to their own circumference of curvature.

2. The magnitude of the forward tangential thrust imparted to the body depends on (*a*) the magnitude of the internally generated bending couples, and (*b*) the form of the waves. If friction operates on the surfaces of external restraint the thrust also depends on the coefficient of lateral friction and on the position of the restraints.

3. From a mechanical point of view, an undulating organism (irrespective of its size and internal structure) can be regarded either as a series of curved levers or as a series of inclined planes.

4. The general principles of undulatory swimming are the same as for a terrestrial glide, except for the fact that each element of the body must possess a component of motion normal to its surface if it is to contribute towards the propulsion of the animal; this type of motion can only occur when the waves move backwards relative to the ground. The animal cannot move forward as fast as the waves are propagated over the body.

5. The propulsive powers of three-dimensional waves are limited to the extent to which the organism is restrained by external forces from spinning about its own longitudinal axis. Otherwise the principles of progression are the same as for two-dimensional waves: the resultant of all the forces acting normally to the body is equal but opposite to that of all tangential forces.

DURING undulatory movement the body of the animal exhibits a train of waves which—with a few interesting exceptions (Gray, 1939)—move, relative to the head, in a direction opposite to that in which the organism moves relative to the ground. This type of motion is found over a wide range of the animal kingdom and over a very wide diversity of bodily size and internal anatomy. In its most intriguing form the movement is displayed by filaments, less than a micron in diameter, whose internal structure must probably be visualized on a molecular level; at the other end of the scale are relatively large animals (e.g. snakes) of known anatomical structure which nevertheless exhibit movements of the same fundamental nature as the

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tail of a spermatozoon. The main object of this paper is to consider the movement of all such systems, in the light of certain general principles common to them all, in the hope that such an inquiry may ultimately throw light on the functional significance of the fibrils and other structures, revealed by the electron-microscope, in a microscopic flagellum.

Irrespective of size and internal structure, all undulatory organisms present two main problems: (i) the nature of the internal mechanism which generates the train of waves passing over the body; (ii) the relationship of the form and frequency of the periodic changes in bodily form to the force driving the animal forward. So long as the undulatory system is relatively large and is exerting its effort against a rigid environment neither of these problems presents any insuperable experimental difficulty (Gray, 1946; Gray and Lissmann, 1950), but when the organism is very small and is exerting its effort against water, a relatively simple exercise in rigid mechanics is transferred to the much more difficult field of hydrodynamics (Taylor, 1951; 1952, *a* and *b*). From a physical standpoint the treatment given in the ensuing paper lacks both elegance and precision, but has, possibly, the advantage of being more readily intelligible to the majority of biologists. The paper should be regarded as a generalized and somewhat over-simplified background against which experimental and observational data will be reviewed in subsequent papers.

I

(i) *Two-dimensional undulatory progression under rigid environmental conditions*

In terrestrial animals undulatory progression can be regarded as an adaptation for movement over or through a relatively rigid medium. Four typical examples cover a wide range of size, speed, and internal structure: snakes gliding between stones; arthropod larvae, e.g. *Ceratopogon*, creeping over sand grains; nematode worms creeping between small intestinal villi or through soil; protozoa, such as *Cristaspira*, creeping through the wall of the crystalline style of an oyster. In all these cases, each part of the animal's body moves along a sinuous path and each part follows the same track as that of its neighbours. The essential features of motion can be readily visualized by enclosing a flexible but inextensible cylinder (or the body of a dead snake) within a close-fitting sinuous glass tube (fig. 1); each part of the cylinder is passively bent to fit the tube, and the lateral curvature of each region differs from that of the region next anterior or posterior to itself. If the cylinder is subjected to an external forward tractive force, the degree of lateral curvature of each region is continuously changed to that previously characteristic of the region next anterior to itself, and a bending wave is transmitted posteriorly over the

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FIG. 1. Diagram showing the variation in lateral curvature of seventeen elements (*A–Q*) of an undulating body enclosed within a rigid tube. The mean curvature measured towards the left side varies from $+1$ to -1. The relative curvature of each element is shown by the numeral on the left side; the centre of curvature of each element is shown by the corresponding small letter (*a–n*). If the cylinder is drawn forward in the direction of the arrow, each element glides forward about its instantaneous centre of curvature, and its radius of lateral curvature changes to that previously possessed by the element next anterior in the series.

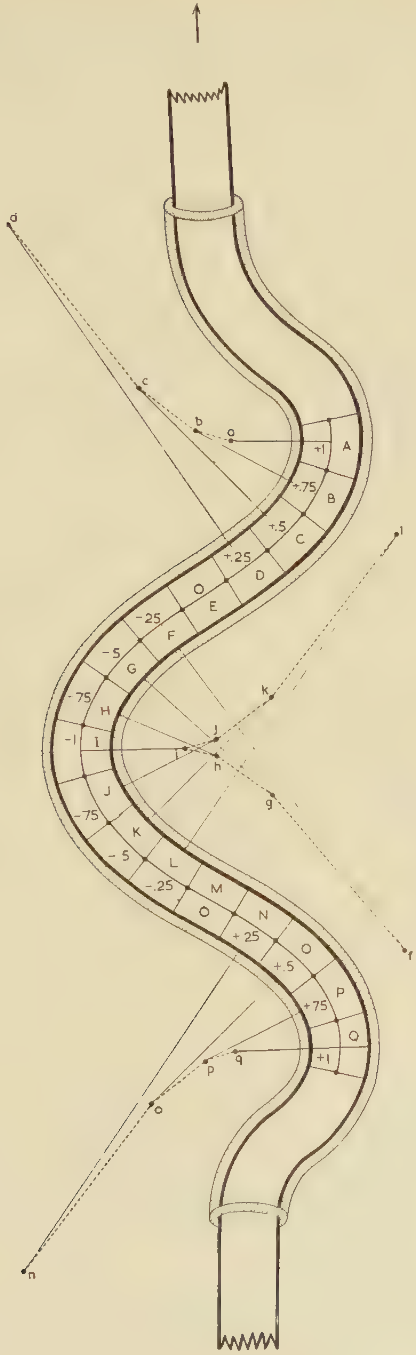


FIG. 1 (*see opposite*).

cylinder at the same speed as that at which the cylinder is being draw forward through the tube. The changes in form undergone by the cylinde and its motion relative to the tube are the same as those exhibited by a livin animal when gliding actively through a tube of similar shape. In both case the form of the wave depends on two characteristics of the external environment: (i) the maximum degree to which the tube is bent at its crests (i.e. o the minimum radius of curvature of the tube); (ii) the difference in curvatur between adjacent regions of the tube. Physiologically these characteristics o the wave depend on the maximum degree of shortening exhibited by laterally situated tensile elements and on the difference in length of successiv such elements along the sides of the body.

The passage of a wave along the body of an organism is an expression of th fact that each element of the body is constantly changing its form toward that previously characteristic of an element adjacent to itself (see fig. 1). I the change is towards that of the element next anterior to itself, the wave travels antero-posteriorly; if the change is towards that of the next posterio element the wave travels postero-anteriorly, i.e. towards the head of the animal. If either the maximum degree of curvature, or the phase interva between adjacent elements, varies in successive elements along the length of the body, the form of the wave changes as it travels along.

In order that each element of the body should undergo these periodic changes in lateral curvature it must be subjected to appropriate bending couples. In vertebrate animals the source of these couples is obvious: the longitudinal axis of the body is rendered incompressible by a chain of rigid vertebrae and, consequently, any muscular tension developed unilaterally about this axis induces compression of the vertebral axis. Equal but opposite bending couples are thereby developed within the element concerned. In arthropods the function of the vertebral column is performed by the exoskeleton; in annelids compression of the axis is resisted by the hydrostatic pressure of the body fluid. Compression elements must also exist within a protozoon flagellum although their nature is, as yet, obscure. In all cases, however, the changes in form undergone by the body of an undulating organism are the result of internally generated bending couples.

(ii) *The mechanism of propulsion*

As all parts of an undulating organism move forward with uniform speed along the sinuous path of motion, all tangential forces (e.g. friction) exerted on the body by the external environment retard its motion; they cannot drive the animal forward. The forward propulsive thrust necessary to initiate or maintain progression must be derived from external forces acting normally to the surface of the body. From a mechanical point of view an analysis of undulatory progression is essentially concerned with the mechanism whereby localized internally generated bending couples expose the organism to a pattern of external forces, acting normally to the surface of the body, whose resultant is equal but opposite to the tangential forces of restraint.

The cycle of deformation undergone by each element of the body during the passage of a 'two-dimensional' bending wave is illustrated in fig. 2; tensile elements, lying laterally to a more centrally situated axis of compression,

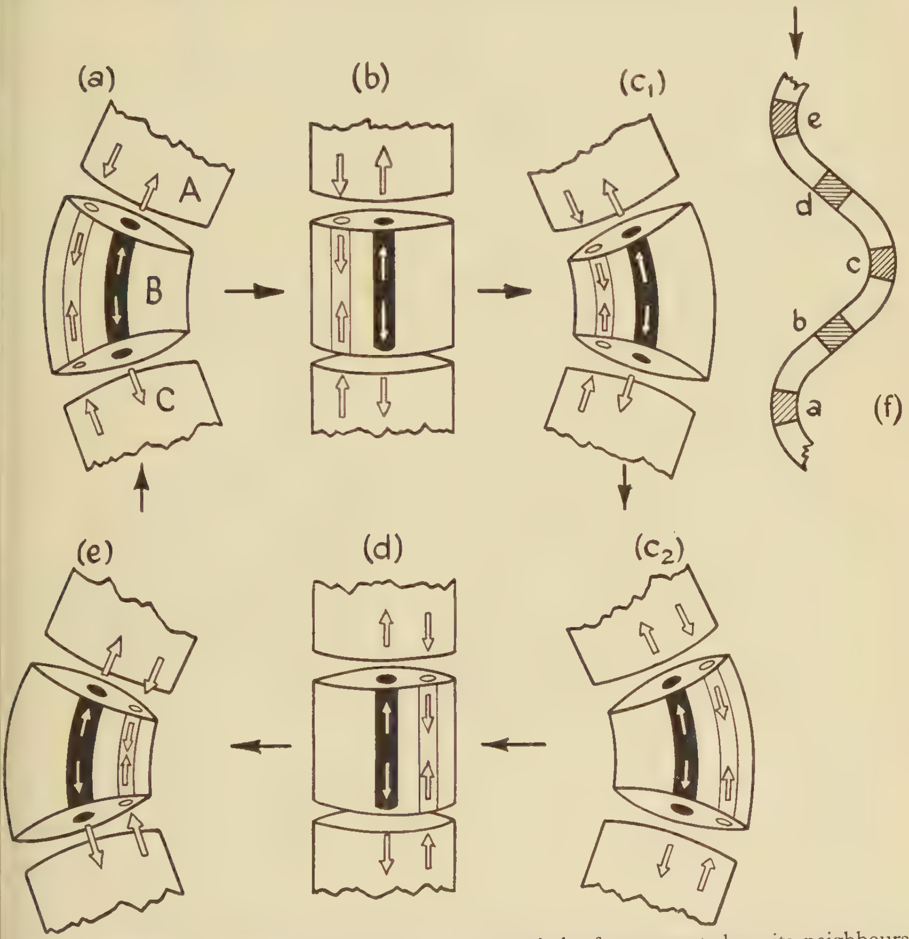


FIG. 2. Diagram illustrating the changes in form and the forces exerted on its neighbours (*A* and *C*) by an actively bending element (*B*). Figs. *a–e* indicate successive phases of one complete bending cycle. In *a* the tensile elements of the left side are beginning to shorten and the central axis develops compression; equal and opposite turning couples are exerted on the neighbouring elements *A* and *C*. In c_1 the tensile elements of the left side are fully contracted and at this stage those of the right side come into action (c_2). The position of the phases *a–e* along the wave is shown in *f*, where the arrow indicates the direction in which the wave of contraction is passing along the body.

undergo changes in length. Equal but opposite bending couples are thereby generated within the active element, and corresponding turning couples are applied to the elements lying anteriorly and posteriorly to the bending element (fig. 2). *As a prime mover of propulsion an actively bending element generates a propulsive thrust, provided it exerts its effort against neighbouring*

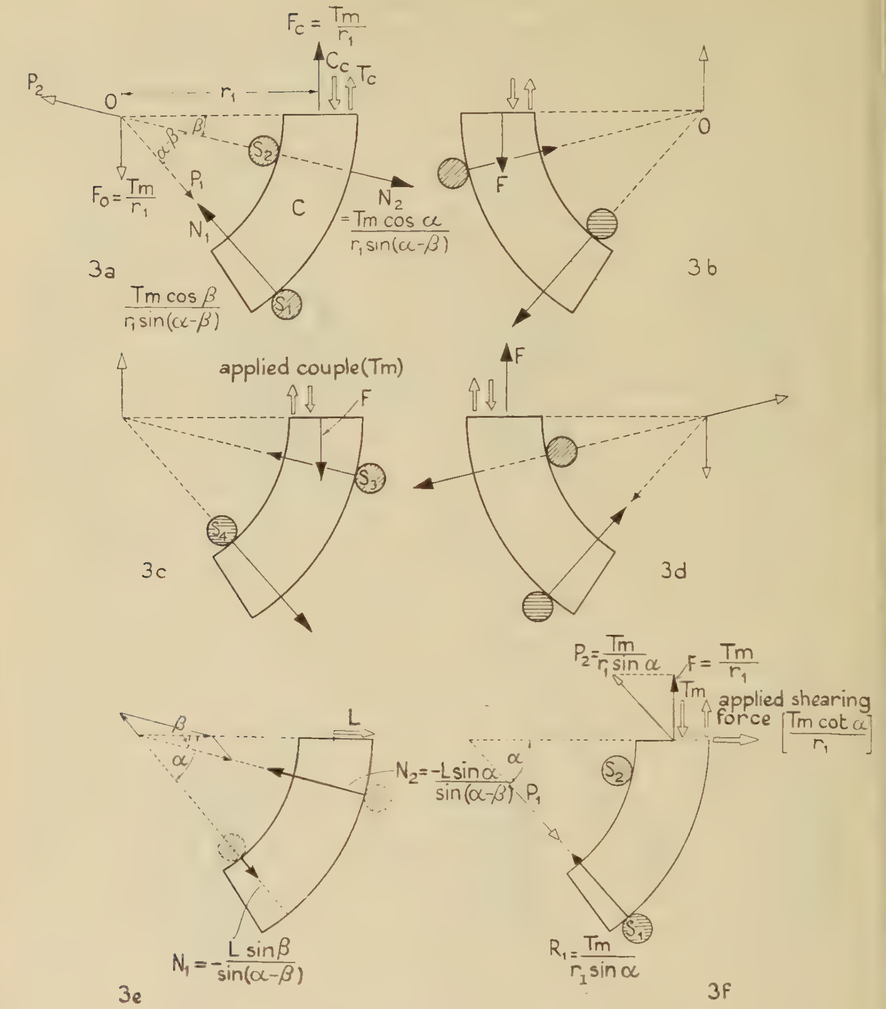

$F_c = \frac{Tm}{r_1}$
C_c
T_c
P_2
O
r_1
β
α
P_1
S_2
$F_0 = \frac{Tm}{r_1}$
C
$N_2 = \frac{Tm \cos \alpha}{r_1 \sin(\alpha - \beta)}$
N_1
3a
$\frac{Tm \cos \beta}{r_1 \sin(\alpha - \beta)}$
S_1
O
F
3 b
applied couple(Tm)
F
S_3
S_4
3c
F
3d
L
β
α
$N_2 = -\frac{L \sin \alpha}{\sin(\alpha - \beta)}$
$N_1 = -\frac{L \sin \beta}{\sin(\alpha - \beta)}$
3e
$P_2 = \frac{Tm}{r_1 \sin \alpha}$
$F = \frac{Tm}{r_1}$
Tm
applied shearing force $\left[\frac{Tm \cot \alpha}{r_1}\right]$
α
P_1
S_2
S_1
$R_1 = \frac{Tm}{r_1 \sin \alpha}$
3f

FIG. 3. When a turning couple (C_c, T_c) of moment Tm is applied to a curved element (radius r_1) in contact with two rigid restraints (S_1 S_2), the resultant is an axial driving force $\frac{Tm}{r_1}$ exerted at the end of the element. The applied couple is equivalent to a backward force (F_0) acting through the centre of curvature O, and a propulsive force of equal magnitude acting at the end of the rod; the force F_0 elicits, from the restraints, reactions whose resultant is equal but opposite to itself. The rod can be regarded as a lever whose fulcrum O is fixed by the operation of the restraints. The direction of the propulsive thrust is reversed if the rod is curved towards the opposite side (fig. 3, *b*) or if the sign of the applied couple is reversed (fig. 3, *c*).

A lateral shearing force (L) applied to the end of the rod elicits reactions from the restraints (fig. 3, *e*) but does not affect the propulsive thrust derived from an applied couple. For a critical value of shear $\frac{Tm \cot \alpha}{r_1}$ the reaction from one restraint becomes zero (fig. 3, *f*); alternatively, if a couple is applied to an element which is in contact with one restraint only, the element exerts, at its anterior end, a shearing force as well as a propulsive thrust.

units which conform with three conditions: (i) the units lying anteriorly and posteriorly to the actively bending element must differ from each other in their radius of curvature; (ii) both units must be restrained from moving normally to their own axes by the presence of external restraints; (iii) both units must be sufficiently rigid to resist the shearing stresses imposed on them by external forces. These essential principles are illustrated in figs. 3 and 4.

In fig. 3 the lateral surfaces of a unit C, of radius of curvature r_1, are in contact with smooth, rigid external restraints S_1, S_2. If an external turning couple (T_c, C_c) of moment Tm is applied to the anterior and (by means of an actively bending element comparable with B in fig. 2) the mechanical effect is expressed in the form of a forward axial thrust $\left(F_c = \frac{Tm}{r_1}\right)$ and an equal but opposite force (F_0) acting through the centre of curvature of C at O. The latter force elicits from the restraints (S_1, S_2) reactions (N_1, N_2) whose resultant is equal but opposite to F_0. The net effect of the applied couple is therefore the axial thrust $F\left(=\frac{Tm}{r_1}\right)$. As shown in fig. 3, *a–d*, the direction of the thrust depends on the sign of the moment of the applied couple about the centre of curvature; hence the direction of the thrust is reversed either by a reversal of the side towards which the element is bent (fig. 3, *b*), or by a reversal of the side of the element on which the tensile elements are active (fig. 3, *c*). Since an actively bending element must apply equal but opposite turning couples to the units lying anteriorly and posteriorly to itself it follows that a net propulsive thrust can only develop when the forward thrust derived from a posterior unit is greater than the backward thrust from the anterior unit or vice versa; in other words, when the radius of curvature of unit A (figs. 2 and 4) is greater or less than that of C; the net propulsive thrust being $Tm\left(\frac{1}{r_1} - \frac{1}{r_2}\right)$. This important conclusion is illustrated in fig. 4 where an actively bending element B (of radius r_2) lies posteriorly to an element A (of radius r_3) and anteriorly to an element C (of radius r_1) $r_3 > r_2 > r_1$; the system is in contact with six external resistances. If element B exerts its active effort against its neighbours entirely in the form of turning couples $(T_a, C_a; T_c, C_c)$ it elicits from A and C, the forces F_a and F_c (fig. 4, *b*) whose resultant has a small lateral component (which elicits a reaction from restraint S_3) together with a propulsive force $Tm\left(\frac{1}{r_1} - \frac{1}{r_3}\right)$ turning B about its centre of curvature at p; the reactions from S_1, S_2, S_5, and S_6 are determined as in fig. 3, *a–d*.

It may be noticed that the axial propulsive force elicited from the system is independent of any shearing effect which element B may exert on its neighbours or vice versa; but, as shown in fig. 3, *e*, *f*, these forces influence the reactions which develop from the individual restraints and thereby alter the bending moments of these forces about each cross-section of the elements A and C. In fig. 4, *c*, the shearing forces operating across the surfaces of contact of the three elements have the limiting value defined by fig. 3, *f* and only three restraints (S_1, S_3, S_5) are operative. In the

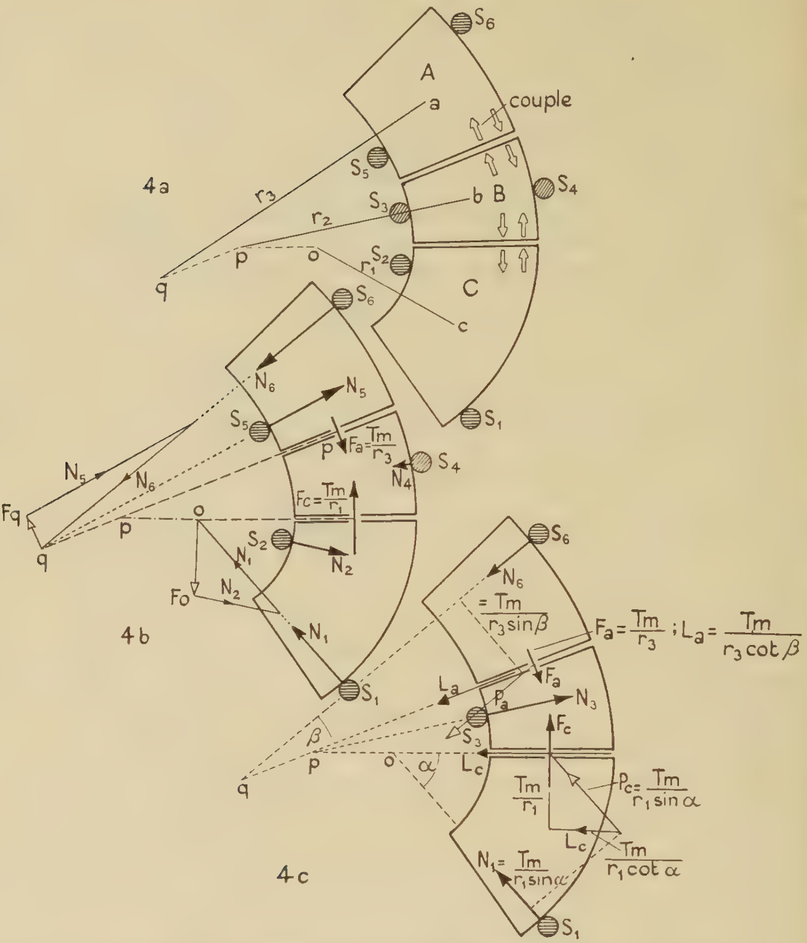


FIG. 4, *a*, an element *B* of average radius r_2 (*bp*) lies posteriorly to an element *A* of radius r_3 (*aq*) and anteriorly to an element *C* of radius r_1 (*co*). It is in contact with six smooth rigid restraints (S_1–S_6).

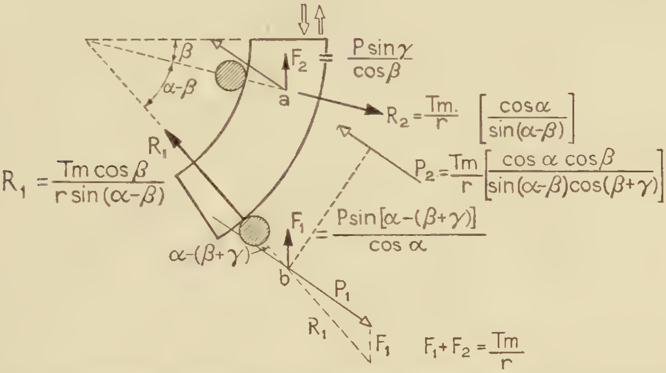
FIG. 4, *b*, if element *B* develops internal bending couples on its right side without exerting any shearing forces across its surfaces of contact with *A* and *C*, element *B* is subjected to a forward axial thrust $\left(F_c = \frac{Tm}{r_1}\right)$ from *A* and a backward thrust $\left(F_a = \frac{Tm}{r_3}\right)$ from *C*; these forces exert a small outward force against S_4, and a forward axial thrust $\left(\frac{Tm}{r_1} - \frac{Tm}{r_3}\right)$ rotating element *B* about its centre of curvature *p*. The reactions from S_1, S_2, S_5 and S_6 are determined as in fig. 3.

FIG. 4, *c*, if element *B* develops its bending couples when the system is restrained by S_1, S_3, and S_6 only, the surfaces of contact between *B* and its adjacent elements must resist shearing forces (L_a; L_c); the axial thrust applied to *B* is however unaltered.

(In this figure the reactions from the restraints are shown on half-scale.)

animate system the lateral bending stresses operating on A and C are determined y the positions of the external restraints; but, in a living system free to operate gainst more than three restraints, the reaction from each restraint is determined y the relative tension of adjacent series of tensile elements and not vice versa.

In fig. 4 the whole of the propulsive energy is derived from element B, or only this element undergoes an active change in shape. Element B is the rime mover. The elements A and C can be regarded as rigid propellers; heir lateral tensile elements responding passively and exerting sufficient orce to enable A and C to resist bending under the forces exerted on them y element B and by the external restraints with which they are themselves


β
α-β
$F_2 = \frac{P \sin\gamma}{\cos\beta}$
a
$R_2 = \frac{Tm.}{r}\left[\frac{\cos\alpha}{\sin(\alpha-\beta)}\right]$
R_1
$R_1 = \frac{Tm \cos\beta}{r \sin(\alpha-\beta)}$
$P_2 = \frac{Tm}{r}\left[\frac{\cos\alpha\cos\beta}{\sin(\alpha-\beta)\cos(\beta+\gamma)}\right]$
$F_1 = \frac{P \sin[\alpha-(\beta+\gamma)]}{\cos\alpha}$
α-(β+γ)
b
P_1
R_1
F_1
$F_1 + F_2 = \frac{Tm}{r}$

IG. 5. Diagram illustrating the action of surfaces of contact as inclined planes. The applied ouple is equivalent to forces (P_1, P_2) acting at the points (a, b) of intersection of the reactions rom S_1 and S_2 with the line of the propulsive thrust. The forces P_1 and P_2 act obliquely to the ine of the reactions from S_1 and S_2; the sum of their propulsive components (F_1 and F_2) is $\frac{Tm}{r}$ and the values of R_1 and R_2 are the same as in fig. 3, a, where the rod can be regarded as lever pivoted at its centre of curvature.

n contact. In a living organism there is no such division between prime mover nd propeller; all elements of the body change their shape and all transmit, o each other, forces which enable the whole system to exert its effort against he outside world.

Although the energy required to maintain a forward propulsive thrust is derived from internally generated bending couples, it is usually more convenient to define the propulsive thrust as the resultant of the external forces cting normally to the surface of the organism. Each element of the body can e regarded as a curved lever; in fig. 3 the centre of curvature O can be regarded s a rigid fulcrum fixed by means of the external restraints S_1 and S_2. Alternatively, the surfaces of restraint can be regarded as inclined planes (see fig. 5) and the system regarded as a 'two-dimensional screw'.

During steady motion the propulsive thrust which is the resultant of all the forces acting normally to the body must be equal but opposite to the total angential drag. A snake gliding through a rigid glass tube or *Cristaspira* reeping through the wall of the crystalline style of an oyster can be regarded s a two-dimensional screw of variable pitch driven through a rigid nut by

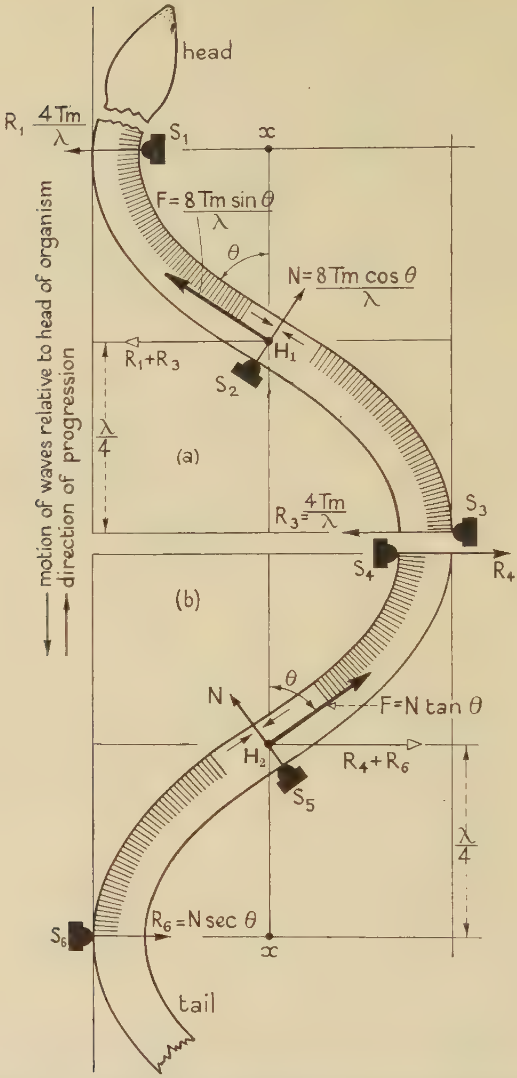


FIG. 6. Two half waves of wavelength λ, and cutting the axis (xx) of progression at an angle θ, are each restrained by three restraints (S_1–S_3 and S_4–S_6).

Fig. 6, *a*, shows the value of the tangential driving force (F) and the reactions (N, R_1–R_6) from each restraint in terms of the maximum couple developed about H_1 and H_2 (opposite S_2 and S_5) by the lateral tensile units; the shaded areas show the side of the body on which the tensile units are active. If Tm is the moment of the couple operating opposite S_2 or S_5, the tangential propulsive thrust is $\frac{8Tm \sin\theta}{\lambda}$ and the reaction (N) from S_2 is $\frac{8Tm \cos\theta}{\lambda}$.

Fig. 6, *b*, shows the value of the propulsive force and reactions from S_4 and S_6 in terms of the reaction (N) from S_5; $F = N \tan\theta$. The driving force is the resultant of the three external reactions (R_4, N, and R_6). If the two half-waves operate as a unit, the reactions from S_3 and S_4 become zero, and equivalent shearing forces operate between the two half-waves.

an internal source of energy. A simple model is provided by a short length of flat spiral spring in contact with three smooth nails driven into a board; if the spring is drawn backward against the restraint of the nails, the curvature is altered between the points of restraint; the spring glides forward as soon as it is released.

(iii) *The relationship of propulsive thrust to wave-form*

As each half-wave of an undulating organism is characterized by progressive change in curvature along its length, it can operate as a single propulsive unit when actuated by the tensile structures lying on the side towards which the curvature is increasing anteriorly, successive half-waves being actuated by tensile structures on opposite sides of the body. As the animal glides along, all the elements constituting a half-wave change their shape and can thereby contribute towards the propulsive thrust. The relative contribution of each of the elements concerned could be determined either from a knowledge of the tension developed at various stages in the shortening of the tensile units, or from a precise knowledge of the distributions and magnitude of the forces acting normally to the body. Neither of these requirements can, as a rule, be satisfied experimentally and further analysis involves two assumptions: (i) that a fully stretched or fully shortened tensile unit has zero tension; (ii) that the tension developed by each unit rises until half of the shortening process has occurred and then falls again to zero, the rise and fall of tension being symmetrical. So long as the system conforms to these requirements the propulsive thrust F, acting tangentially to S_2 (fig. 6, *a*), can be expressed in terms of the maximum tensile couple (Tm) exerted by any one element, the wave-length (λ) of the wave, and the angle (θ) at which the wave cuts the axis of progression (xx); the tangential thrust (F) is $\frac{8Tm\sin\theta}{\lambda}$ where Tm is the moment of the bending couples operating about the mid-point (H_1) of the system. Alternatively, the tangential thrust can be expressed in terms of the forces acting normally to the body (fig. 6, *b*).

A series of half-waves, similar in form and tensile effort, is shown in fig. 7. External resistances operate at the two ends of the system and against the posterior face of each half-wave. The strain on the tensile units (indicated in the figure by shading lines) is greatest at points situated on the main axis (xx) of progression and falls to zero at the troughs and crests of the waves where the shearing forces reach their maximum value. Each of the external forces (N) operating against the posterior surface of a half-wave (see fig. 7, *b*) has two components (fig. 7, *b*) (i) a driving force ($N\sin\theta$) acting along the axis (xx), (ii) a transverse (yy) component ($N\cos\theta$). During steady movements $\sum N\sin\theta$ is equal but opposite to the backward components ($\sum L\cos\theta$) of the tangential drag (L) acting against the ventral surface of each segment of the body; the resultant of all the transverse forces

$$(\sum N\cos\theta+\sum L\sin\theta+R_1+R_2)$$

is zero.

As seen in fig. 8 the value of θ increases with the amplitude and decreases with the length of the waves; consequently, the tangential thrust (F) to be derived from any particular level of maximum bending effort (Tm) will vary

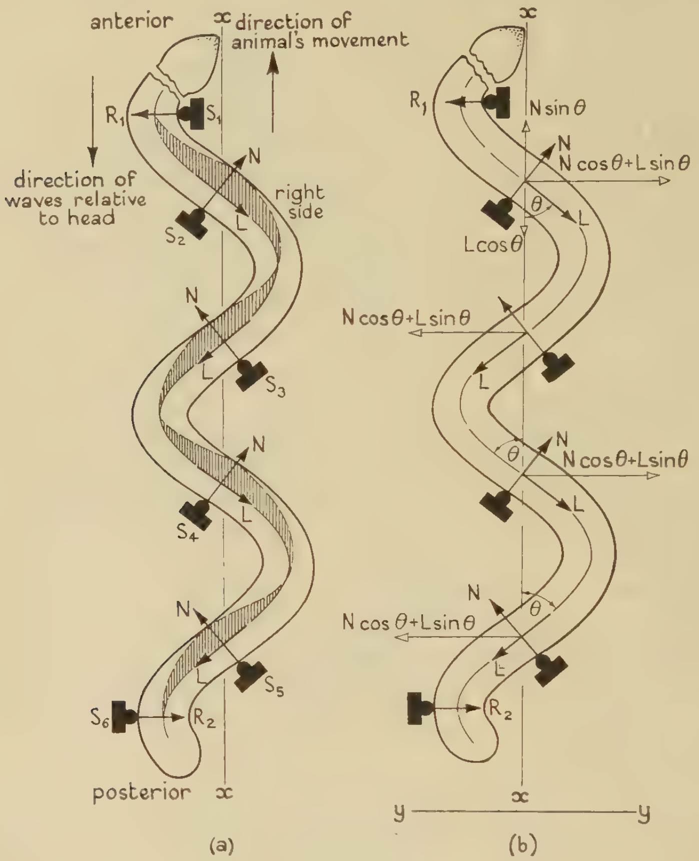
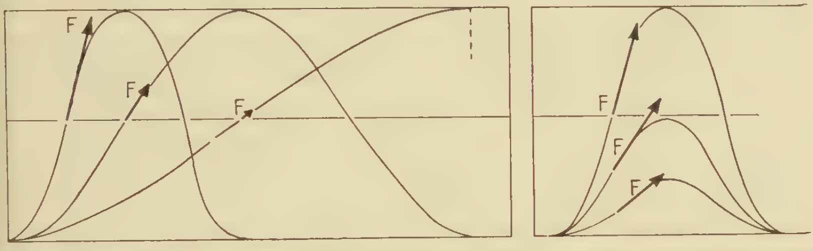


FIG. 7. Diagram showing the general equilibrium between the normal and tangential forces acting on an undulatory organism.

In fig. 7, *a*, the resultant of all the forces (R_1; N_1–N_4; R_2) acting normally to the surface of the animal is equal but opposite to that of all the tangential forces (L); the latter forces being equal but opposite to the propulsive thrusts shown in fig. 6. The shaded areas show the distribution of active tensile units; the width of the area shows the relative degree of tension of the tensile units.

Fig. 7, *b*, the forces (N) acting against the reverse faces of the waves have components ($N \sin \theta$) acting forwards along the axis (xx) of movement and transverse components ($N \cos \theta$); the drag forces have backward components ($L \cos \theta$) acting along the axis xx, and transverse components ($L \sin \theta$); during steady motion $N \sin \theta = L \cos \theta$, and the resultant of all the transverse forces is zero.

accordingly. Waves of large amplitude and short length may be regarded as adaptations for overcoming high frictional resistance between the ventral surface of the body and the environment. Similarly, if the surfaces of contact between the sides of the body and the external resistances are not perfectly nooth the forward tangential thrust (F) becomes $\frac{8Tm\sin\theta}{\lambda}(1-\mu\cot\theta)$ here μ is the coefficient of lateral friction; if $\mu = \tan\theta$ no driving thrust is ailable—in other words, the organism would have to increase the pitch of e waves in order to propel itself forwards. On the analogy of vertebrate uscles in general, maximum undulatory efficiency may be expected to occur hen the extent and rate of shortening of the tensile units (i.e. the form of the aves) bears a particular relationship to the tangential resistance to be overome.



IG. 8. Diagram illustrating the relationship between the tangential axial thrust (developed y couples of equal maximum strength) to the wavelength and amplitude of the bending wave. The thrust decreases with wavelength and increases with amplitude.

II

(i) *Two-dimensional undulatory movement in water*

Just as an undulatory organism gliding through a close-fitting and rigidly xed sinuous tube can be compared with a screw moving in a rigid nut, so the motion of the animal in water can be compared with the same screw when perating against a fluid resistance. Alternatively, the swimming organism an be regarded as gliding through a glass tube which slips backwards elative to the ground as the organism moves forward. Neither in the case f a swimming organism nor a screw propeller is it possible to provide a omprehensive picture of the whole system.

Under terrestrial conditions each element of the animal's body is constrained to move along its own circumference of curvature. The external esistances, being rigidly fixed to the ground, are able to exert forces normal o the surface of the body without allowing any part of the body to move ormally to its own longitudinal axis: the animal moves forward, relative to he ground, at the same speed as the waves travel backwards along the body. When swimming in water, on the other hand, forces acting normally to the body can only develop against an element of the body when the latter possesses component of motion normal to its own longitudinal axis—in other words, when the element is moving with its surface inclined by an appropriate angle) to its path of motion. As shown in fig. 9, this fundamental requirement mplies that the waves move backward relative to the ground; this backward movement or 'slip' of the waves (as measured along the axis (xx) of proression) is the most distinctive feature of all undulatory swimming organisms rrespective of size and internal structure; it is, in fact, a measure of the extent to which each element of the wave has a component of motion norm to its own longitudinal axis and thereby is able to elicit a normal reaction fro the surrounding water. The greater the normal component of motion (relativ to the tangential component) the greater is the 'slip' of the waves and vic versa. Since the rate of the animal's forward progression is equal to the difference between the speed of propagation of the waves over the body and th velocity of the 'slip', it follows that the maximum speed of forward progressio for any aquatic undulating organism must be less than that at which the wave are travelling backwards relative to the head of the animal.

The balance of forces required to maintain the steady forward propulsio of an undulatory organism in water is of the same fundamental nature as tha in a terrestrial animal, namely, the retarding effect of all tangential force must be balanced by the propulsive effect of all the forces acting normally t the body. Determination of these forces is beyond the range of direct observation and further analysis is largely restricted to an attempt to express the rat at which an organism can propel itself through the water in terms of th frequency and form of the waves which it propagates along its body. A inquiry of this type encounters very formidable difficulties but a usefu qualitative picture of the propulsive properties of a wave can be obtaine by visualizing the forces which operate on a single element of the body at particular phase of its movement, and considering later the extent to whic it is possible to deduce the propulsive properties of the whole organism as th integrated effect of all its constituent elements.

The force exerted on an element as it moves through the water depends o the size and form of the element and on the speed and direction of its movement relative to the surrounding water. So long as the surface of the elemen is inclined to its path of motion, the resultant force has two components, on (N) normal to the surface of the element and the other tangential (L) to thi surface. If the element is moving with velocity Q with its surface inclined t the path of motion by an angle i,

$$N = C_D(Q), \qquad L = C_L(Q),$$

where C_D and C_L are coefficients which vary with the size and form of the element, its absolute speed, and with the angle i; if i is zero, C_D is zero and there is then no propulsive force.

The propulsive or retarding action of both normal and tangential forces on the movement of the element along the main axis (xx) of propulsion can be visualized by expressing the velocity and direction of the element's motion through the water in terms of its displacement in respect of the main axes (xx, yy) of propulsion and of the angle at which the element is inclined to the axis (fig. 10). If the element is inclined to the xx-axis at an angle θ and is moving in respect to this axis at velocity V_x and along the yy-axis at velocity V_y, the angle of incidence (i) of the surface to the path of motion is such that

$$\tan i = \frac{V_y - V_x \tan\theta}{V_x + V_y \tan\theta}.$$

The angle of incidence has a maximum value ($90^\circ = \theta$) when the element has no component of forward motion ($V_x = 0$) and approaches zero as V_x approaches $V_y \cot \theta$.

The coefficient (C_D) of normal resistance increases with the value of i, hence the force acting normally to the element has a maximum value when V_x is zero and falls to zero as V_x approaches $V_y \cot \theta$. In order to yield a resultant propulsive force along the axis of progression, the propulsive component of N must be greater than the retarding component of L; in other words, $N \sin \theta > L \cos \theta$; and, during forward motion at a steady speed, $L = N \tan \theta$ whilst the resultant of all the transverse (yy) forces is zero. The conditions are thus fundamentally the same as under terrestrial conditions (see figs. 7, 9).

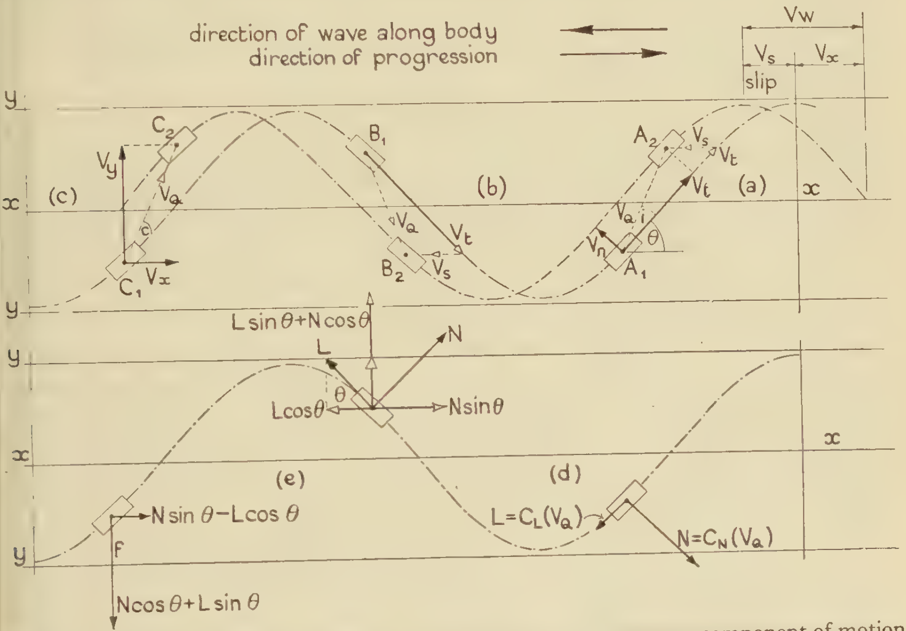


FIG. 9. To exert a forward propulsive thrust an element must possess a component of motion normal to its surface and, consequently, the waves must move backward relative to the ground.

Fig. 9, *a*, the resultant displacement (V_Q) of an element moving with normal velocity V_n and a tangential velocity V_l is the same as for an element moving with a tangential velocity V_t and a backward velocity (V_s) in respect to the *xx*-axis. V_s is the velocity of 'slip' of the waves; if V_n is zero, V_s is zero. If V_w is the speed of conduction of the waves, the forward speed of the animal (V_x) in respect of the *xx*-axis is $V_w - V_s$.

Fig. 9, *c*, the motion of an element moving at a velocity V_Q (inclined at an angle i to its path of motion) expressed in terms of transverse (V_y) and forward (V_x) components.

Fig. 9, *d* and *e*, the element elicits a reaction ($N = C_N(V_Q)$) from the water normal to the surface and a reaction [$L = C_L(V_Q)$] tangential to its surface. The propulsive thrust in respect of the *xx*-axis is $N \sin \theta - L \cos \theta$, whilst the transverse force is $N \cos \theta + L \sin \theta$.

An application of these principles to a whole cycle of an element's transverse movement and to the propulsive properties of the organism as a whole involves known values of C_L, C_D, θ, V_x, and V_y for all phases of the cycle and pre-supposes that V_y can be expressed in terms of the velocity (V_w) at which the waves pass along the body. In a recent paper Taylor (1952) has considered the case of an organism projecting sine waves of known speed, amplitude, and wavelength, and applied values of C_D and C_L derived from wires set at an angle to a wind; he has shown that the expected ratio of the forward propulsive speed V_x to the speed of the waves V_w increases with the amplitude of the waves, and decreases with their wavelength. At the same time, this ratio increases with the absolute size and speed of the organism concerned. An application of the theory to a snake, leech, and ceratopogonid larva shows a very fair measure of agreement between observed and calculated values (see Taylor, 1952 *b*, fig. 4).

Although the above treatment may yield a useful general picture of the mechanical principles involved during undulatory swimming, its application to living organisms must be carried out with extreme caution. At least three limitations must be borne in mind. (i) In few, if any, swimming organisms do the propagated bending waves maintain constant amplitude and wavelength as they pass along the body. Two serious complications thus arise: (*a*) the rate of propagation (V_w) of the waves is not constant, (*b*) the transverse velocity (V_y) and the angle of inclination (θ) of an element at any given phase of the cycle is not the same for all elements. (ii) The theoretical analysis assumes that the water in contact with each element of the body can be regarded as at rest relative to the ground and not affected by the activity of neighbouring elements. (iii) The theory neglects the special conditions which exist at the two ends of the system.

Further consideration of some of the difficulties referred to above will be given elsewhere. For the moment it is possible to draw only four main conclusions. (i) The mechanical principles involved during two-dimensional undulatory swimming are fundamentally the same as those used during a terrestrial glide except for the fact that any element which contributes towards the propulsion of the body must possess a component of motion normal to its own longitudinal axis and thus move with a positive angle of attack between its own surface and its path of motion. Each element functions as an inclined blade or hydrofoil—eliciting from the water a reaction normal to its surface. The propulsive component of this reaction along the axis of progression overcomes the retarding component of the tangential forces acting on the body. (ii) As an undulating organism swims forward the waves passing down the body must move backwards relative to the ground and the maximum rate of the animal's progression must be less than the rate at which the waves are propagated backwards over the body. (iii) The propulsive power of a wave may be expected to increase with an increase of amplitude and decrease with an increase of wavelength (Taylor, 1952 *a*). (iv) The ratio of speed of progression to speed of conduction of waves may be expected to be greater in large animals than in small ones (Taylor, 1952 *b*).

(ii) *Three-dimensional undulatory movement*

Whereas the bending movements exhibited by relatively large animals (snakes, eels, polychaete worms) can be regarded as restricted to a single lane, those of some small aquatic organisms—notably certain Protozoa—are ore complex. Accurate observation of such movements is by no means easy; is important not to underestimate their complexity or overlook the coniderable degree of versatility displayed by a single individual organism under ifferent environmental conditions.

Geometrically, most if not all the complex movements observed in protooan flagella by Ulehla (1911) and other authors, can be described in terms of system wherein the plane of bending changes as the contraction wave passes rom one element to another. If the change in the plane of bending followed n appropriate pattern, the system would acquire the form of a regular helix, nd the form of the waves would resemble those which pass along a rope when ne end is rotated in a circle. How far the movements of any living organism onform with this regular pattern is open to serious doubt, but the mechanical rinciples involved have an interesting bearing on the mechanism of movement f such organisms as spirochaets and spirilla, and, possibly, some bacteria.

A static helical form could be impressed on an elongated structure in two ways: i) by *uniform* bending in one morphological plane, accompanied by an appropriate ngle of torsional displacement between adjacent elements; (ii) by simultaneous inusoidal bending in two planes normal to each other; i.e. by combining 'lateral' ending with 'dorso-ventral' bending and maintaining a constant phase difference f one-quarter of a complete cycle between the two. These two systems are illustrated y fig. 10 where the resultant helical 'organisms' are shown circumscribing imaginary ylinders. The external form of the two systems is identical, but the type of internal eformation of the original filaments is different. From a dynamical point of view nly the potentialities for changes in *external* form need be considered.

There appear to be only two methods by which a helical system could ropel itself. (i) By acting as a passive rigid screw rotated by an external ouple; according to Metzner (1920) this type of motion is exhibited by *Spirillum volutans*, where the requisite couple is provided by terminal flagella. No change in form is exhibited by the elements of the body itself. (ii) Each lement of the body should undergo cyclical changes in form. It is the latter ossibility which is of immediate interest.

If each element of a helical system is regarded as a short cylinder, its ontribution towards the propulsion of the body depends on its ability to hange, by internal effort, the radius of curvature of each of its main morphoogical surfaces; the radius of curvature of its 'dorsal', 'ventral', and 'lateral' urfaces must be constantly changing. If, as in fig. 10, the organism is isualized as in contact with a circumscribed cylinder, it must—if it is to be apable of self-propulsion—be able to bring each of its morphological urfaces in turn into contact with the surface of the cylinder. No animal could rogress if it kept the same surface in contact with the cylinder, for under such onditions there would be no change in the form of its constituent elements nd, consequently, no source of propulsive energy.

In their simplest form the changes in shape undergone by each element f a self-propelling helical system are illustrated by fig. 11, *a*, where the element possesses four tensile elements (*Dorsal, Left, Ventral, Right*) arrange round a central axis of compression. In fig. 11, *b* an element (*B*) of this typ lies between two rigid rods (*A*, *C*); *if one of these* (*e.g. C*) *is rigidly fixed*, th other (*A*) sweeps over the surface of a cone when the tensile units of *B* shorten either in the order *Dorsal, Right, Ventral, Left* (or *Dorsal, Left, Ventral, Right*)

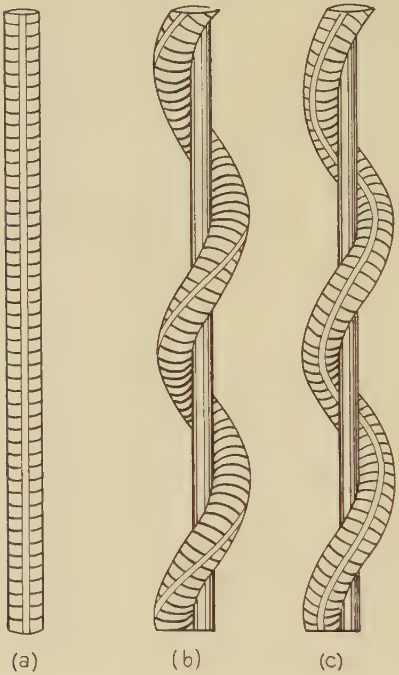


FIG. 10. A uniform cylinder (fig. 10, *a*) can be converted into a helical form either (i) by uniform bending in one plane accompanied by torsion (fig. 10, *b*) or (ii) by simultaneous sinusoidal bending in two planes normal to each other (fig. 10, *c*). The external form of the two systems is the same but the pattern of internal deformation differs. Note that in fig. 10, *b*, the original dorsal surface of the undeformed cylinder (marked by the white line) is of uniform curvature and passes spirally round the central axis of the body; in fig. 10, *c*, the dorsal line is of varying curvature and does not pass round the body.

A series of active elements differing from each other by a constant difference in the phase of its contractile cycle would assume the form of a regular helix circumscribable round a cylinder; as a contractile cycle passes round each individual element, the element would (since it is restrained from spinning about its own central axis) move round the cylinder in a direction opposite to that in which the process of shortening sweeps round the element's tensile units (see fig. 12). The changes in form undergone by each element are essentially those exhibited by each short length of a flexible rope when drawn, *without torsion*, through a rigid helical tube; as an element moves forward through the tube each of its 'morphological' surfaces (Dorsal, Right, Ventral, Left) is in turn bent to fit the 'inner' surface of the tube and during each omplete bending cycle the element moves in a circle round the surface of the cylinder about which the helical tube can be circumscribed.

It is essential to note that a rotary displacement of each element about a ircumscribed cylinder (or about the axis of symmetry of the helix) only

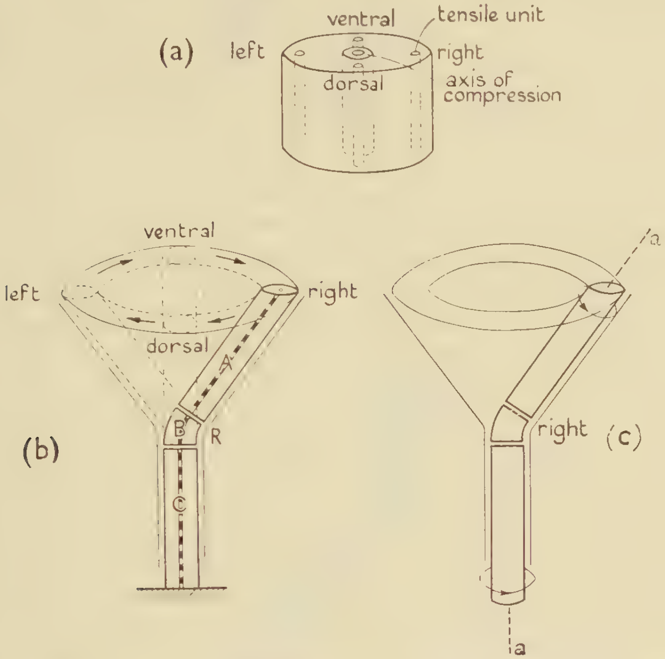


FIG. 11, *a*, a contractile element possessing four tensile units (Dorsal, Left, Ventral, Right) arranged round a central axis of compression; the tensile units being able to shorten in the order Dorsal, Left, Ventral, Right (or *L*, *V*, *R*, *D*). In fig. 11, *b*, an element (*B*) of this type operates against two other rigid elements *A* and *C*, the element *C* being restrained from rotation about its own central axis.

As shown in the figure, the right tensile unit is shortened and the three elements are viewed from the dorsal surface.

If the tensile elements shorten in the order Dorsal, Left, Ventral, Right, element *A* sweeps over the surface of a cone in the direction shown by the arrows in fig. 11, *b*. If, as in fig. 11, *c*, the same contractile cycle occurs when element *A* is not restrained from rotation, the three elements spin about their central axes *aa* without displacement of *A* or *C* relative to the circumference of the cone. Note that the direction of spin is opposite to that in which element *A* moves round the cone in fig. 11, *b*. In fig. 11, *b*, the dorsal surface (shown as a dotted band) of the elements would remain uppermost during the whole contractile cycle; in fig. 11, *c*, the dorsal surface would only be visible during one-quarter of a cycle.

occurs if the element—and therefore the body as a whole—is prevented from spinning freely about its own median axis. In the absence of such restraint each element can undergo its cycle of bending movements without moving its position relative to the surface of a circumscribed cylinder or the walls of a rigid helical container. Thus in fig. 11, *c*, the same bending cycle is occurring as in fig. 11, *b*, but the anterior end of the element does not sweep round the surface of a cone; it remains in the same position spinning about the axis

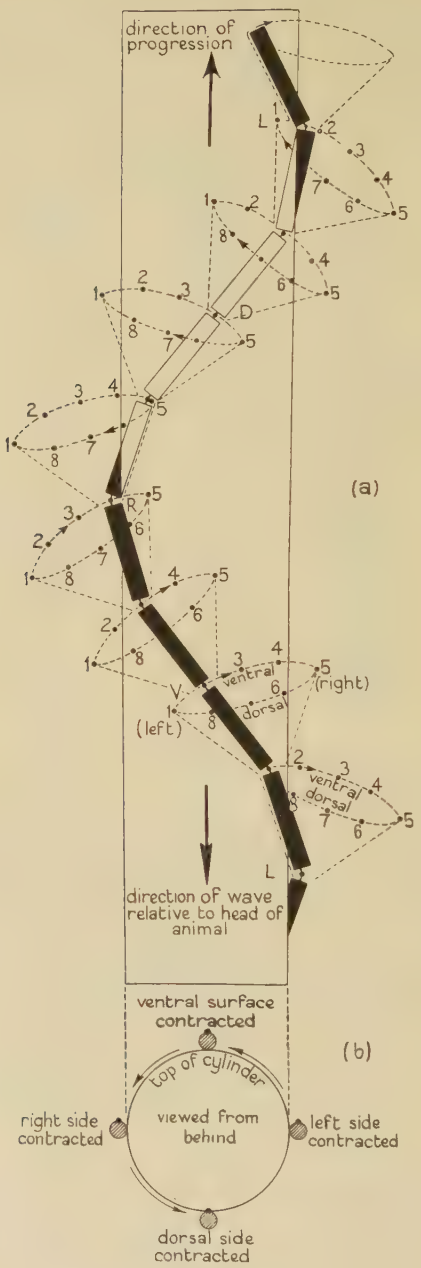


FIG. 12 (*see opposite*).

a in the opposite direction in which it would, if prevented from spinning, sweep round the cone (or round a circumscribed cylinder). From purely geometrical considerations it follows that an organism could, by developing appropriate trains of dorso-ventral and lateral bending waves, propel itself forward along a rigid helical container provided the walls of the container can prevent the body spinning on its own helical axis.

From a dynamical standpoint the basic principles of helical motion are fundamentally the same as those of a two-dimensional system; during steady motion the forward components of forces acting normally to the body must equal the retarding components of all tangential forces. The difference in the two systems lies in the fact that whereas the lateral components of all normal and tangential forces of a two-dimensional wave summate to zero, those of a helical wave yield couples tending to rotate the body in the opposite direction to that in which the elements are moving round the median axis of motion (fig. 13). As shown in fig. 14 any such rotation reduces the speed at which the elements move relative to the environment; if the organism spins through 360° during the passage of each complete wave of bending, the wave would remain stationary relative to the ground and possess no propulsive power. From a functional standpoint helical waves would possess their maximum propulsive power when one end of the system is rigidly fixed—as in the case of a flagellated cell forming part of an epithelium. In such a case the rotating couple set up by the flagellum would be neutralized by forces exerted by the fixed environment. In freely swimming systems, however, some amount of 'spin' is inevitable. Its effect is to reduce the effective speed at which the waves travel relative to the environment, and since the latter factor determines the magnitude of the propulsive thrust, the speed of progression must inevitably be less than when all spin is eliminated. If the frequency of spin were equal to the frequency of propagation of the waves down the body, the waves would remain stationary relative to the medium and no forward thrust would develop; on the other hand, if there is no 'spin' the rate of travel of the waves relative to the medium would be equal to the rate at which they are travelling relative to the head of the organism minus the speed at which the latter is moving forward through the water. Again, the propulsive thrust would be greatest in the case of a flagellum attached to a fixed cell and less in the case of a free-swimming organism; in the latter case the thrust would be increased if the flagellum were propelling a flat ellipsoidal cell or (possibly)

FIG. 12. A series of elements similar to that shown in fig. 11 differing from each other by one-eighth of a contractile cycle form a helix circumscribable about a cylinder. If each element changes its form to that characteristic of the element next anterior to itself whilst the whole body is restrained from spinning on its own axis, each element passes round the circumscribing cylinder once during each contractile cycle.

The motion of the body relative to the circumference of the cylinder is identical to that of a rope drawn forward (in the direction of the top arrow) *without torsion* through a close-fitting helical tube of similar form to the series of elements shown in the figure. The large letters *L, V, R, D, L* indicate the location of the fully shortened tensile elements.

In fig. 12, *b*, the dorsal side of the body is marked by a black dot; the shortened elements are located at the surface of body in contact with the cylinder.

if the flagellum itself were elliptical in cross-section. If the frequency of generation of the waves were x per second and the body is spinning about the axis of motion $x-n$ per second, the frequency of the waves relative to a fixed point in the environment would be n per second and the organism would, owing to 'slip', advance about a helical path whose pitch is less than that of the waves and it would complete n turns of this helix per second. As noted by Reichert (1909), the existence of spin is highly characteristic of many flagellated Protozoa, and the above discussion confirms that author's conclusion concerning its mechanical origin.

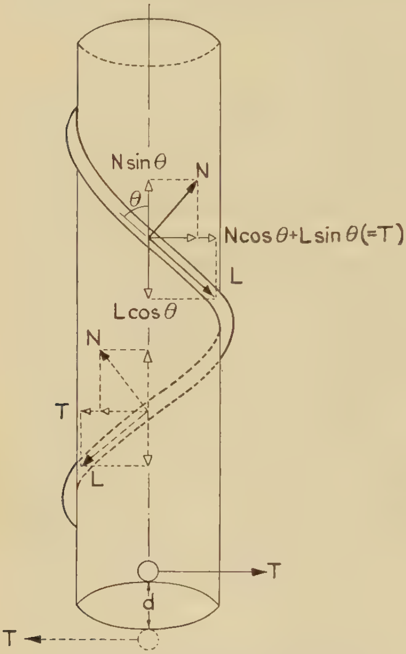


FIG. 13. In a regular helical system all the forces (normal and tangential) exerted on a wave act tangentially to the surface of a cylinder. For any one element the normal force (N) has a forward component $N \sin \theta$ and a transverse component $N \cos \theta$; the tangential drag (L) has a backward component $L \cos \theta$ and a transverse component $L \sin \theta$. For an element situated one half of a wavelength from the first, the total transverse force will, with that of the first element, yield a couple turning the elements and the body as a whole about the axis of the cylinder in a direction opposite to that in which the elements are moving round the cylinder. During steady motion $N \sin \theta = L \cos \theta$, whilst the 'spinning' couple is $d.L \operatorname{cosec} \theta$, where d is the diameter of the helix.

The motion of a *regular* helical system, relative to the surrounding medium, would (if the system be prevented from spinning as a whole about its long axis) be identical with that of a rigid screw of the same helical form when rotated, by an external couple, at a frequency equal to that at which the bending waves are being propagated along the length of the actively undulating organisms. The two systems could only be distinguished from each other by observation of a point marked on the surface of the body. If the body were opaque, a point visible at any phase of motion of the active flagellum would be visible at all others, whereas a similar point on a rigid screw would only be visible for half of each cycle. The essential difference between the two systems lies in the fact that each element of the active organism is constantly changing its shape, whereas no change occurs in a passively rotated rigid screw. The motion of the organism through the water depends on its ability to execute these changes in shape against the resistance of the surrounding medium.

Further consideration of so-called 'helical' organisms will be given elsewhere but two general points may be mentioned here. First, an examination of the behaviour of grass snakes in the light of the above analysis has revealed a somewhat striking fact. These animals can glide through sinuous tubes lying in a vertical plane with the same facility as that displayed through horizontal tubes. During a 'vertical' glide the animal uses its dorso-ventral musculature and uses its lateral muscles when gliding horizontally. There is, therefore, no anatomical reason why it should not negotiate a helical tube of equivalent pitch and diameter. All attempts to elicit a helical glide have however failed; one is tempted to wonder whether the animal has failed to evolve a type of muscular co-ordination which, in the absence of any 'anti-rotation' device, would be of no functional value! It would be interesting to know whether a snake with an elliptical cross-section could, on the other hand, negotiate a regular helix of similar cross-section. Second, it should be borne in mind that the existence of a 'self-propelling' organism of regular helical form is by no means too well established: most three-dimensional waves are probably elliptical in cross-section and not circular, and in such forms the spinning couple imparted to the body is correspondingly reduced.

A mathematical derivation of the magnitude of the spinning couple operating on a self-propelled helical system has recently been given by Taylor (1952 *a*).

III

Notes on observational data

So long as an animal is gliding against rigid restraints the application of the principles described above does not encounter any obvious difficulties. In large animals the equilibrium between the forces acting normally and tangentially to the body has been established experimentally (Gray and Lissmann, 1950) and there is no reason to suppose that smaller forms, less amenable to experimental analysis, should behave differently. On the other hand, the observation of organisms swimming in water reveals data which indicate the limitations of any existing theoretical analysis. For the sake of convenience, attention will be restricted for the moment to relatively large organisms (eels, snakes, and ceratopognid larvae); nematodes and unicellular systems will be dealt with in separate communications.

The form of the waves passing down the body of an anguilliform fish has been described elsewhere (Gray, 1933). The animal exhibits rather more than one complete wavelength and the amplitude of transverse displacements increases as the wave passes posteriorly. The changing form of the moving wave is conveniently expressed in terms of the body's displacements relative to fixed axes through the head of the animal; the area which includes the body during one complete muscular cycle being regarded as its characteristic envelope. In an anguilliform fish this envelope is approximately an isosceles triangle with its apex at or near the anterior end of the body. The same general characteristics are revealed by a snake (*Natrix natrix*) swimming in water. Photographic records reveal two features of the snake's movements which are not covered by the theoretical analysis. (i) Both amplitude and wavelength are approximately doubled as a wave crest moves between a point situated about one-quarter of the body length from the head to a point one quarter of the body length from the tip of the tail; the rate of propagation (V_w) of the wave therefore increases as the wave travels backward. (ii) The

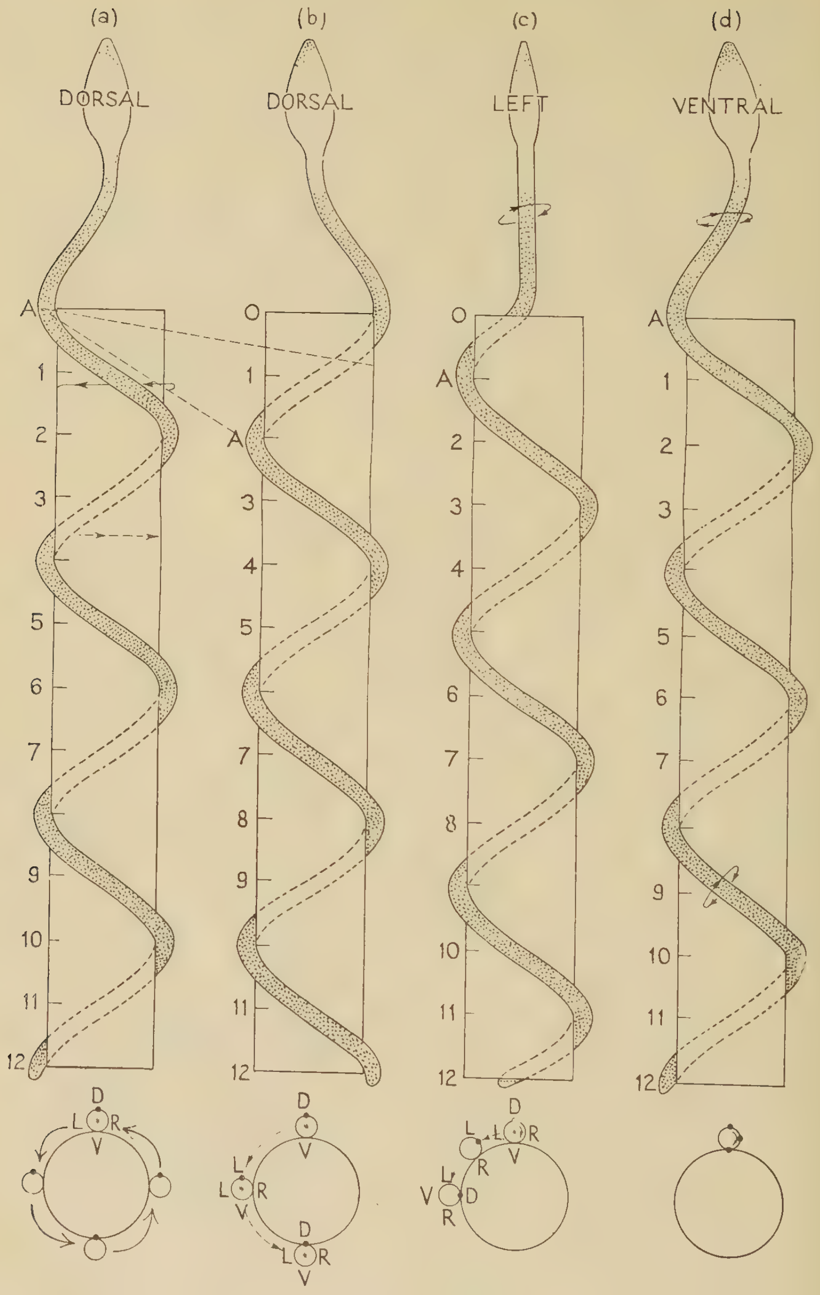


FIG. 14 (*see opposite*).

orward speed (V_x) of the animal's progression is relatively constant. Conequently, the ratio $\frac{V_x}{V_w}$ varies during the passage of the wave; towards the osterior end of the body the ratio is of the order 0·4–0·5, whereas at the front

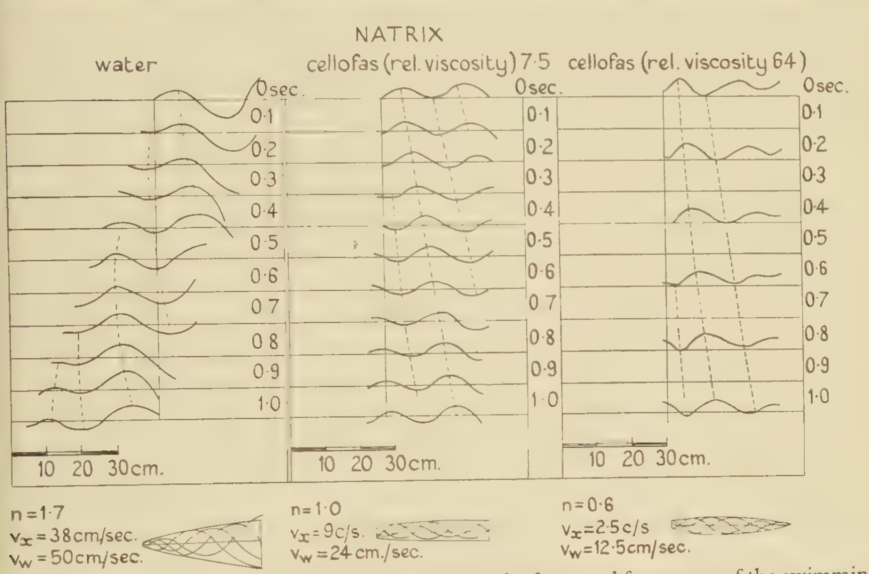


FIG. 15. Diagram illustrating the effect of viscosity on the form and frequency of the swimming movements of a snake (*Natrix natrix*).

end of the body the ratio may be unity or even more; the *average* value being 0·7–0·8 (see Taylor, 1952 *b*).

The extent to which the form of a wave changes as it passes along the body depends on the nature of the external fluid. As shown in fig. 15, these characteristics vary with the viscosity of the medium. In a sufficiently viscous medium the amplitude progressively *decreases* as the wave passes posteriorly, the tip of the tail exhibiting little or no transverse movement. The facts

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FIG. 14. Diagram illustrating the effect of axial spin on the movement of helical waves. The movement of the waves is indicated relative to a smooth cylinder which maintains a constant distance behind the head of the animal. The head of the animal is flattened dorso-ventrally. Dotted areas show regions of the body lying over the cylinder. The wavelength is four units.

In fig. 14, *a*, the organism is at rest and presents its dorsal surface to the observer; the most anterior wave crest (*A*) is at zero on the cylinder. If each element of the body executes one half of a complete contractile cycle whilst the body is completely restrained from spinning, each wave crest travels half-wavelength backwards along the cylinder (fig. 14, *b*); if the organism as shown in fig. 14, *a*, spins through 90° whilst undergoing one-half of a contractile cycle, the wave crests only travel one-quarter of a wavelength as in fig. 14, *c*; if the body spins 180° during each half-contractile cycle, the wave crests remain stationary as in fig. 14, *d*.

Note that for any given element the phase of contraction is exactly the same in figs. 14, *b–d*, viz. half-cycle ahead of that in fig. 14, *a*; in fig. 14, *b*, the organism still presents its dorsal surface to the observer at the end of the half-cycle of contraction, whereas it presents its left surface in fig. 14, *c*, and its ventral surface in fig. 14, *d*. In each case the direction of 'spin' is in the direction opposite to that in which each element of the system moves round the circumscribed cylinder.

indicate that the speed and form of the waves are determined by externa mechanical forces.

The contrast in form (and frequency of movement) between a creepin and a swimming ceratopogonid larva is even more marked than those of snake; on transference to water the frequency of the larva's movements i greatly increased, and the extremities of the body exhibit relatively littl bending. As shown in fig. 16, the animal moves forward spasmodically as th

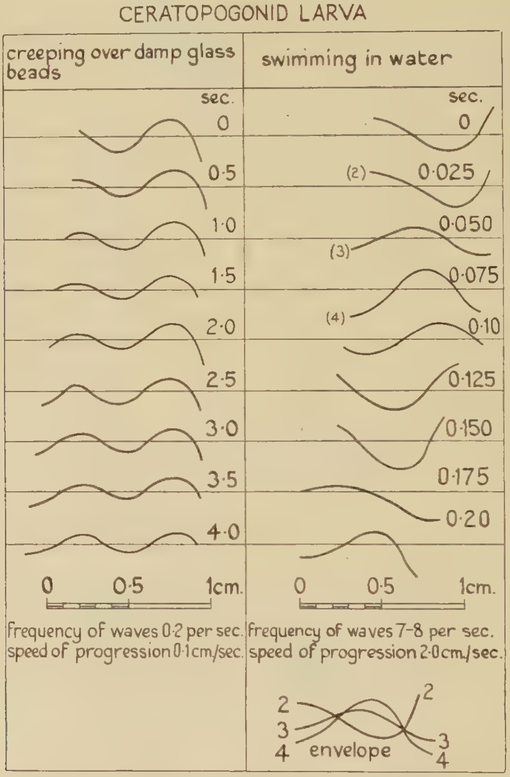
CERATOPOGONID LARVA
creeping over damp glass beads
swimming in water
sec.
0
0·5
1·0
1·5
2·0
2·5
3·0
3·5
4·0
sec.
0
(2)
0·025
0·050
(3)
0·075
(4)
0·10
0·125
0·150
0·175
0·20
0
0·5
1cm.
0
0·5
1cm.
Frequency of waves 0·2 per sec.
speed of progression 0·1cm/sec.
Frequency of waves 7–8 per sec.
speed of progression 2·0cm/sec.
2
3
4
envelope
2
3
4

FIG. 16. Diagram illustrating the form of movement of a Ceratopogonid larva when creeping over a solid surface and when swimming freely in water.

posterior end of the body sweeps across the axis of progression and remains relatively stationary whilst the waves pass over the front two-thirds of the body. The ratio of average speed of progression to the speed of propagation of the wave is relatively small, and since the frequency of the movement is of the order of 7–8 per second the form of the 'envelope' is visible to the eye of the observer. This envelope is shown diagrammatically in fig. 16, and exhibits two well-marked 'nodes'. Envelopes of this type clearly differ fundamentally from those of any system propagating a train of waves of constant form, and it is open to doubt how far such living systems can, at present, be subjected to satisfactory theoretical analysis. Well-defined nodal nvelopes are exhibited by certain nematodes, but for the moment it is nteresting to note that they can readily be elicited from an eel placed in air n a smooth level table. In the latter case the displacements of the body elative to the table are in conformity with the assumption that the centre of ravity of the body remains fixed. The phenomenon of nodal envelopes will e further discussed elsewhere, but there can be little doubt that they are etermined by the nature of the external medium, and confirm the conclusion hat the frequency and form of the bending cycles exhibited by each element f the body of an undulating organism are determined by extrinsic mechanical orces.

As indicated earlier in this paper, undulatory movement appears in its most interesting form in structures such as flagella or the tails of many permatozoa. Many of these forms are only a small fraction of a micron in diameter and it is of interest to consider the types of problems which they aise in the light of the general principles discussed above. These problems re three in number. Firstly, the movements exhibited by the flagellum are n expression of internally generated bending couples, and, consequently, he flagellum must possess both tensile and compression elements. If the ibres observed in 'preserved' specimens are a reliable guide to the structure f the living flagellum, are some of these tensile and others compression lements, and if so, how are they arranged in the living system? Secondly, what is the mechanism which controls the extent of the contraction of the ensile elements and the difference in phase between adjacent elements? Is it necessary to postulate some form of 'neuroid' transmission from element to lement, or is the form of the wave entirely determined by elements which contract as soon as they are exposed to a particular pattern or intensity of external mechanical forces? Thirdly, each element of the flagellum must be ed with sufficient potential energy to maintain its own rhythmical motion. The nature of this essential part of the mechanism, like the others already alluded to, must be sought by methods comparable with those used for a physiological analysis of undulatory motion in larger organisms; they are unlikely to be revealed by static observation of dead cells by means of microscopes—electronic or otherwise.

Thanks are due to a number of colleagues—to Dr. T. Gold, Dr. H. W. Lissmann, and Lord Rothschild in particular—for their interest in this problem and for the many valuable suggestions made by them during the progress of the work.

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